UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENICAS QUÍMICAS
Departamento de Bioquímica y Biología Molecular

TESIS DOCTORAL

Estudio de xilanasas fúngicas para el aprovechamiento de la biomasa lignocelulósica

Study of fungal xylanases for the exploitation of lignocellulosic biomass

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Madrid, 2018

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A mi abuelo Manolo

A mis padres

Tranquilo tío, somos científicos

Bill Murray (como el Dr. Peter Venkman), Los Cazafantasmas

ACKNOWLEDGMENTS

This work has been carried out with funding from projects PRI-PIBAR-2011-1402, RTC-2014-1777-3, BIO2015-68387-R from MINECO and RETO-PROSOT S2013/MAE-2972 from Comunidad de Madrid. Manuel Nieto thanks the MINECO for his FPU fellowship and the Department of Biochemistry and Molecular Biology I (Faculty of Chemistry, Universidad Complutense de Madrid) for the teaching training.

El que aquestas palabras suscribe es tristemente consciente de que, en el espacio que se le ha concedido, difícilmente podrá dar cumplida cuenta de todos aquellos que se han hecho merecedores de su gratitud durante estos venturosos cuatro años. Mas su honra empeña en que intentará dar a cada uno lo que en justicia le correspondiere y si alguno se perdiere en el tintero de esta torpe pluma, sepa que aunque la razón haya fallado en el corazón permanece.

En primer lugar no puedo sino dar las gracias a la directora de esta tesis, la Dra. María Jesús Martínez, gran científica y mejor persona. Te doy las gracias por confiar en mí todos y cada uno de los días que he pasado en tu laboratorio, por reírte con mis exposiciones (sin despedirme luego), por tu optimismo a prueba de ataques nucleares, por tu guía y ánimos, por enseñarme hacer las cosas bien y por tu mágica habilidad para solucionar el problema más complicado en el último minuto.

En co-primer lugar gracias a la Dra. Laura de Eugenio, con toda probabilidad la mejor codirectora que haya existido jamás, considerando el planeta Tierra y varios universos paralelos. Dueña de mi destino, en la salud y en la enfermedad, hasta que la tesis nos separe, pero amiga para siempre. Gracias por tu humor negro y tu cariño incondicional pobremente disimulado. Gracias por enseñarme que aunque las cosas no hayan salido del todo bien, siempre hay alguien que te compra la burra. Gracias por la tarta Guinness y hasta por el heavy metal (no, eso es broma). En general, gracias por todo Lidem.

Gracias a todos mis compañeros de Biotecnología para la Biomasa Lignocelulósica, ¿quién iba a pensar que detrás de un nombre tan aburrido hubiera tan buena gente? Gracias a Jezú, embajador de Barbate en el mundo y mi cicerone en el laboratorio. Gracias por tu buen humor, porque contigo aprendí que aunque los experimentos no salgan, el Madrid o Nadal acaban por ganar algo y por supuesto gracias por incumplir siempre tu amenaza de pegarme. Gracias pisha. Gracias a Mariu, fuente y destino de toda información. Mi vecina de poyata, compañera de tantas penas y alguna alegría. Gracias por tu paciencia, por enseñarme lo que es un salseo en condiciones y ante todo por estar siempre ahí, dispuesta a ayudarme cada vez que lo necesitaba. Gracias Davichúa Salvachúa por tu alegría

contagiosa y tu sabiduría enciclopédica. Aprendí de ti desde el día que llegué hasta el que te fuiste a vivir el sueño americano. Gracias a Peces, Rosa Peces, insultantemente joven, pero fichaje estrella del laboratorio. Gracias por ser tan buena gente, por poner orden en el caos cuando nos quedamos sin Mariu (sólo han pasado cuatro líneas y ya te echo de menos otra vez Mariu), por tus ganas de reír y en definitiva por ser un sol. Gracias a Juanillo (A.), hermano de hongo. Gracias por esos cafés de avellana y ciencia, por tu coche siempre listo para una misión de los Manolos, por ser un Caballero de la Lamprea e inasequible al desaliento. Que sepas que si no te para tu tobillo no te parará nadie. ¿Y qué decir de Fran? Pues que llegó a mi vida como una ola de fuerza desmedida y cargada de medios, tampones y placas siempre a punto. El cielo es más azul desde que tú estás en el laboratorio y aguanta despejado hasta las cinco de la tarde. No te vayas nunca. Gracias a Úrsula, inventora de la capoeira. Si el 249 fuese una serie tú serías mi personaje recurrente preferido. Gracias a Norge, el Dr. Quorum Sensing. Gracias por recorrer los duros caminos de la ciencia y haber vuelto entero (más o menos) para contárnoslos y gracias por esas reuniones en el Instituto Ondiñas de Investigación de firmes comienzos y borrosos finales. Gracias a María, de personalidad magnética y repostera inigualable (tú también eres inigualable Lidem). Gracias por ser la voz de la razón en el loco país de Nunca Jamás. Gracias a Neumara, perfecto equilibrio entre samba brasileña e ingeniería química. Gracias porque metodología de respuesta en superficie suena (un poco) más ameno con acento tropical. Gracias a la Dra. Ali, co-co-directora en la sombra e ilustre políglota. Gracias por tus ganas de pasarlo bien haciendo ciencia de primera, porque tu buen humor es como la contaminación en Madrid, no hay forma de perderlo y lo más importante, por tener siempre cinco minutos para sentarte conmigo aunque haya una multitud con antorchas en tu puerta. Gracias a Marijose, jefa absoluta del laboratorio, que me enseñó todo lo que un hombre necesita saber (y lo que no necesita también) sobre ligar en el bingo y besos sorpresa.

Cambio de laboratorio y también de párrafo que empezaba a ser demasiado largo. Gracias a Lola Linde López (L^3), capaz de iluminar una habitación con su sonrisa mientras nos intoxica con tolueno. Gracias a Remedios Pacheco, más conocida como Piña, gracias por tu risa perenne y por enseñarme que el arte moderno no es (tan) malo. Gracias a Vero, un ángel con pipetas, en Suecia no saben la suerte que tienen. Gracias a Juanillo (R.), compañero gallego en el exilio, tu cara de sueño y resignación por las mañanas me hacía sentir menos sólo en la vida. Gracias a Ana, exportadora del mañacao, y posiblemente la postdoc más joven de espíritu (lo que no es espíritu quedará en el misterio) que haya conocido. Gracias a Iván, el latin lover de Ávila, gracias por todos esos buenos consejos que nunca seguí sin que dejaras de ser mi amigo. Gracias por las

apuestas absurdas de incierto resultado y sobre todo gracias por convencerme para que, de vez en cuando, disfrutara de la luz solar. Gracias a David, Amanolito para los amigos y ¡Almendral! para los más íntimos. Gracias por ser genio y figure y por liarla lo justo para desviar de nosotros la atención de los jefes. Como sé que leerás esta frase contando las palabras que te he dedicado continuaré alargando innecesariamente el texto, por lo menos una línea más, para asegurarme de que quedas total y absolutamente satisfecho. Gracias Supernieto. Gracias también a Ángel Martínez, sapientísimo doctor y al locuaz Dr. Javi Ruíz Dueñas por mantener los euros de Europa fluyendo, la impresora en marcha y acertar siempre con los licores de sobremesa. Gracias a Falip, Pilaf al revés, alquimista de la anilina y el sumial. Gracias por ser un romántico incomprendido y buen compañero donde los haya. Gracias a Isa (P), encantadora y temible a partes iguales, pero brillante como nadie. Gracias por acoger pacientemente el rol en el salón de tu casa. Gracias a Isa (V), mi amor platónico (platónico de momento) y la persona más luminosa del CIB en particular y del Consejo Superior de Investigaciones Científicas en general. Gracias porque allá donde estás más claros los fluorescentes brillan y los solventes orgánicos se respiran mejor. Gracias a la Dra. Susana Camarero, la ambición rubia y la única persona capaz de hacer divertido hasta un congreso de lignina. Gracias a todos aquellos que en algún momento han pasado por el laboratorio aportándome siempre algo positivo. Gracias a Cris Coscolín, por ser locamente genial (algunos dirían genialmente loca), a Lorea que me descubrió que hay comidas veganas que saben hasta bien, a Erik, el del gorro perenne, a Bea Balcells, Elena, Aitor, Jose, Lucía, los Felipes Pavel, Jacobo, David y Mario (tú también eres un Felipe, Mario y lo sabes) y a alguno más que probablemente me esté olvidando, pero que así podrá echármelo en cara en el futuro.

Sin salir del CIB pero cambiando de planta tengo que dar las gracias a mis compañeros de RMN, los Dres. Javier Cañada y Ángeles Canales y muy especialmente a Bea capaz de resolver un xilósido en menos de 24 horas y de desaparearme los espines con una sola mirada encantadora. Gracias también a los Dres. Antonio Romero y Elena Santillana por ayudarme a asomar la mirada al arduo mundo de la cristalografía de rayos X. Tampoco me puedo olvidar de Blas Blázquez (me podría poner un warning), maestro de las magics, ni de Juan Nogals, el Señor Oscuro que el día menos pensado te puede dar trabajo. Gracias de corazón a Julia, científica excepcional, prometedora jugadora de baloncesto y ante todo amiga. Gracias por ser magnánima cada vez que digo algo inconveniente y por enseñarme a bailar (o por lo menos intentarlo). Gracias a Lola Alonso, mi bioinformática favorita, capaz de recordar el directorio, del log, del último salvado de un proyecto de hace seis meses mientras todos los demás nos quejamos de Changnesia. Gracias a Paco y Vivi de Proteómica y a Leo

de Croma, por su ayuda, sus consejos y sobre todo por su buen trabajo y esfuerzo.

Gracias por supuesto a Carlitos, que se merece su propio punto y aparte. Gracias por ser la mejor persona que haya existido fuera de un santoral y mi (particular) brújula moral. Ha sido un honor tenerte de amigo todos estos años y un placer debatir contigo las ideas más absurdas de las formas más irresponsables. Espero que llegues muy lejos allá donde vayas, para algún día poder ponerte de referee.

Gracias a todos mis amigos y compañeros del ICP, empezando por el Dr. Francisco Plou, que me acogió como uno más de su grupo aunque los HPLCs temblasen cuando me veían entrar por la puerta. Muchas gracias por tu guía y por tu apoyo Kiko. Gracias a Berni, mi viejo compinche del trabajo fin de máster que sabe como nadie darle emoción y riesgo a la hora de comer, a Noa por su paciencia infinita y su retranca galaica, que me hacen sentir casi en casa. Gracias a Lopa, biotecnóloga de confianza. Gracias a Antonio, Javi, Pati, Patri, Dava, Xavi, Cris (sales dos veces Cris) y el resto de ICPeros que me reciben siempre con los brazos abiertos.

Gracias a todos mis compañeros de la estancia canadiense en la UBC, sé que no sabéis español y nunca leeréis esto, pero ahí queda. Gracias al Dr Stephen Withers por aceptarme en su grupo de magníficos y responder a los correos aunque estuviera en Francia. Gracias a Zach que tuvo tiempo para supervisarme mientras estaba siendo padre. Gracias Andrés, español en tierra extraña como yo, químico insuperable y guía turístico aún mejor. Gracias por compartir conmigo buenos momentos en lengua cervantina y seguir colaborando conmigo a la vuelta de la aventura. Gracias a Georg, mi vecino de poyata germano, a Lars, Emily, Feng, con su inglés de acento chino, Hong-Ming, con su chino de acento inglés, Miriam, Spence y todos los demás que me descubristeis ese país maravilloso de temperaturas bajas y salarios altos.

De mi estancia en Valencia quiero dar las gracias a toda la buena gente de Biopolis, en especial a Ester Pardo que fue todo amabilidad cuando le aparecí por sorpresa, cual gatito abandonado. Gracias también a Paqui y a Ginés. Gracias por acogerme en vuestra casa y por estar dispuestos a compartir conmigo vuestros bizcochos y pictogramas epidérmicos.

Quiero dar un agradecimiento especial al inconsciente Rubio, al no tan bravido Federico, al conquistador Gaspark y a la nunca virtuosa Topamí, que llenaron con sus (des)venturas las tardes del domingo.

Gracias a Juan A. Olmeda, no confundir con Olmedo, que sepas que han sido nuestros planes para convertir la ciencia en crujientes euros lo que me ha mantenido (más o menos) cuerdo estos años. Gracias a Hugo, amigo desde el origen de los tiempos. No importa dónde nos veamos porque siempre será como volver a estar en casa. Gracias a Martiña, porque las rías

y la lamprea están bien, pero lo que más mola de Galicia eres tú. Gracias a Emi, por ser mi mejor amigo. Ya sé que se escribe pronto, pero significa mucho. Gracias a Mer, por acogerme en mi peregrinación anual a Berlín. Tú haces que cada visita sea más genial que la anterior.

Gracias a mis abuelos y a mi tío Moncho por su apoyo incondicional (moral y económico) animándome siempre a seguir con "esas cosas de los hongos". Puede que yo haya perdido alguna vez la ilusión en lo que hacía, pero vosotros en mí jamás. Gracias a Elena y a Ramón, por venir a ocupar el sofá de vuestro hermano, año tras año, puntuales como la gripe, pero mucho más gratos. Gracias por escucharme siempre y recordarme que lo importante no es lo difícil que sea el doctorado, sino el sueldo que puedes ganar después (ingenuos). Finalmente, pero no por ello menos importante sino todo lo contrario, gracias a mis padres. Gracias por vuestro apoyo, cariño y ánimos. Gracias por la receta del marisco. Gracias por las veces que os preocupáis con razón y también por todas las que lo hacéis sin ella. Sois científicamente los mejores padres del mundo. No tengo claro si hubiera podido llegar al final de este camino sin vosotros, lo que sé seguro, es que se me habría hecho mucho más cuesta arriba.

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ABBREVIATIONS

NUCLEOTIDES

A	Adenine	**M**	A/C	**B**	C/G/T
C	Cytosine	**R**	A/G	**D**	A/G/T
G	Guanine	**S**	G/C	**H**	A/C/T
T	Timine	**Y**	C/T	**V**	A/C/G
K	G/T	**W**	A/T	**N**	A/C/G/T

AMINO ACIDS

A/Ala	Alanine	**M/Met**	Methionine
C/Cys	Cysteine	**N/Asn**	Asparagine
D/Asp	Aspartic acid	**P/Pro**	Proline
E/Glu	Glutamic acid	**Q/Gln**	Glutamine
F/Phe	Phenylalanine	**R/Arg**	Arginine
G/Gly	Glycine	**S/Ser**	Serine
H/His	Histidine	**T/Thr**	Threonine
I/Ile	Isoleucine	**V/Val**	Valine
K/Lys	Lysine	**W/Trp**	Tryptophan
L/Leu	Leucine	**Y/Tyr**	Tyrosine

ENZYMES

AXE	Acetyl xylan esterase
BxTW1	β-xylodase 1 from *Talaromyces amestolkiae*
CAZyme	Carbohydrate active enzyme
CE	Carbohydrate esterase
Endo H	Endoglycosidase H
FAE	Feruloyl esterase
GH	Glycosyl hydrolase
GT	Glycosyl transferase
PL	Polysaccharide lyase
rBxTW1	Recombinant BxTW1
(r)BxTW1	Native and/or recombinant BxTW1
Rex	Reducing-end-xylose releasing exo-oligoxylanase
XynM	Endoxylanase from *Talaromyces amestolkiae*

NUCLEAR MAGNETIC RESONANCE

^{1}H-^{13}C-NMR	Two-dimensional proton carbón-13 nuclear magnetic resonance
^{1}H-NMR	Proton nuclear magnetic resonance
DOSY	Diffusion-ordered spectroscopy
HSQC	Heteronuclear single quantum coherence
NMR	Nuclear Magnetic Resonance
TOCSY	Total correlation spectroscopy

OTHER

1G, 2G	First generation, second generation
2,6-DHN	2,6-dihydroxynaphthalene
2-ME	2-mercaptoethanol
A_{280}	Absorbance at 280 nm
BBD	Box-Behnken design
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAZy	Carbohydrate active enzyme database
CBM	Carbohydrate binding module
DMSO	Dimethyl sulfoxide
DMEM-F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
DTT	Dithiothreitol
EGCG	Epigallocatechin gallate
ESI	Electrospray ionization
FOS	Fructooligosaccharides
FPLC	Fast protein liquid chromatography
GC	Gas chromatography
Glc	D-Glucose
GOS	Galactooligosaccharides
iFBS	Inactivated fetal bovine serum
K_m	Michaelis constant
k_{cat}	Catalytic constant
k_{cat}/K_m	Catalytic efficiency
k_i	First-order inactivation rate constant
He	Helium
HPAEC-PAD	High-performance anion-exchange chromatography coupled with pulsed electrochemical detection
HPLC	High-performance liquid chromatography
HQ	Hydroquinone
HQX	Hydroquinone xyloside
HT	Hydroxytyrosol
HTA	Hydroxytyrosol acetate
HTX	Hydroxytyrosol xyloside
IL	Ionic liquid
IMOS	Isomaltooligosaccharides
LPS	Lipopolysaccharides
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MeGlcA	4-*O*-methyl-D-glucuronic acid
MS	Mass spectrometry
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NP	Nitrophenol
***o*-**	*orto*-/2-
OD_{600}	Optical density at 600 nm
***p*-**	*para*-/4-

***p*NP**	*p*-Nitrophenol
***p*NP-Arap**	*p*-Nitrophenyl α-L-arabinopyranoside
***p*NP-Araf**	*p*-Nitrophenyl α-L-furanoside
***p*NPG**	*p*-Nitrophenyl β-D-glucopyranoside
***p*NPX**	*p*-Nitrophenyl β-D-xylopyranoside
PCR	Polymerase chain reaction
PDB	Protein data bank
pI	Isoelectric point
RES	Resveratrol
RSM	Response surface methodology
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSF	Simultaneous saccharification and fermentation
TFA	Trifluoroacetic acid
T50	Temperature at which the activity is half the maximal
TLC	Thin layer chromatography
V_{max}	Maximum reaction velocity
X1	Xylose
X2	Xylobiose
X3	Xylotriose
X4	Xylotetraose
X5	Xylopentaose
X6	Xylohexaose
XOS	Xylooligosaccharides

RESUMEN/SUMMARY

RESUMEN

Introducción

El xilano constituye la segunda mayor reserva de carbono de la biosfera, sólo precedido por la celulosa. Es un heteropolisacárido perteneciente al grupo de las hemicelulosas por lo que está presente en la mayoría de las principales fuentes de biomasa lignocelulósica. Estructuralmente, estos polímeros se caracterizan por tener una cadena principal de unidades de D-xilopiranosa unidas por enlaces β-1,4. Este esqueleto presenta frecuentes acetilaciones y está altamente ramificado, con cadenas laterales muy cortas, formadas por residuos de arabinosa o ácido glucurónico. La abundancia de cada uno de los sustituyentes depende en gran medida del tipo de biomasa vegetal. Debido a esta complejidad su hidrólisis necesita de la acción concertada de toda una batería de enzimas, de entre las cuales las endo-β-1,4-xilanasas y las β-xilosidasas desempañan un papel esencial. Las primeras hidrolizan el polisacárido atacando enlaces internos de la cadena principal, liberando como productos oligosacáridos con distinto grado de polimerización. Las β-xilosidasas son enzimas que completan la degradación, convirtiendo estos xilooligosacáridos (XOS) en xilosa.

La importancia económica del aprovechamiento de este heteropolisacárido nace fundamentalmente de su gran abundancia y ha supuesto un fuerte impulso para la investigación sobre ambas enzimas xilanolíticas. La industria busca tanto la sacarificación del xilano, con vistas a la obtención de biocombustibles, como su conversión en productos de alto valor añadido. En este último campo, las endoxilanasas pueden aplicarse para la obtención de XOS, considerados actualmente prebióticos emergentes. En cuanto a las β-xilosidasas, aunque su papel más conocido es el hidrolítico, muchas presentan también la capacidad de transferir un residuo de xilosa a un compuesto aceptor, en una reacción denominada transxilosilación. De esta forma se podrían obtener glicósidos con propiedades bioactivas, abriendo un nuevo campo para la aplicación de estas enzimas.

Las enzimas xilanolíticas son producidas en la naturaleza principalmente por bacterias y hongos, con el fin de degradar los polisacáridos de la pared celular vegetal. De entre estos organismos, son los hongos filamentosos los que han despertado un mayor interés como productores de estas enzimas en los últimos años. Esto es debido a que muestran mayores niveles de actividad frente a xilano y frecuentemente secretan las enzimas de interés al medio extracelular, lo que facilita tanto su purificación como el uso directo de los crudos fúngicos en diferentes aplicaciones.

Objetivos

Talaromyces amestolkiae CIB es un hongo ascomiceto aislado de residuos de cereales, e identificado en el laboratorio de la Dra. María Jesús Martínez en el Centro de Investigaciones Biológicas, donde se ha realizado la presente tesis doctoral. El sistema celulolítico de este hongo había sido caracterizado previamente mostrando propiedades de gran interés. Por este motivo se continuaron los estudios sobre este organismo abordando el análisis de las enzimas implicadas en la degradación del xilano. Para ello se definieron los siguientes objetivos:

a) Estudio de la producción por *T. amestolkiae* de enzimas implicadas en la degradación del xilano.

b) Purificación y caracterización, desde un punto de vista bioquímico y molecular, de las principales enzimas xilanolíticas secretadas por este hongo.

c) Estudio de las posibles aplicaciones biotecnológicas de estos biocatalizadores para la valorización de la biomasa vegetal.

Resultados

Estudio de la producción de enzimas xilanolíticas por T. amestolkiae

Los niveles de actividad xilanolítica alcanzados por *T. amestolkiae* se ensayaron cultivando el hongo en medio líquido en presencia de distintas fuentes de carbono. De entre los potenciales inductores probados, el xilano de haya a una concentración del 2% (p/v) fue el que conllevó la máxima secreción tanto de actividad β-xilosidasa como endoxilanasa. Estas condiciones fueron mantenidas en el resto del trabajo para la producción de las enzimas de interés.

Caracterización de una nueva β-xilosidasa de interés biotecnológico

A partir de los cultivos de *T. amestolkiae*, se purificó y caracterizó una nueva β-xilosidasa. La posterior secuenciación de su gen permitió clasificarla como perteneciente a la familia 3 de las glicosil hidrolasas. La enzima (BxTW1) presentó un grado de *N*-glicosilación en torno al 10% y una estructura cuaternaria dimérica. Su caracterización reveló varias propiedades de interés. A nivel físico-químico la enzima presentó una notable tolerancia a la presencia del catión Cu^{2+}, potente inhibidor de la mayoría de enzimas xilanolíticas y celulolíticas. Su máxima actividad se encontró a valores de alta acidez (pH 3) aunque su estabilidad fue alta en un amplio rango de pH (2-9). Desde el punto de vista cinético, la enzima

destacó por presentar los mayores valores de eficacia catalítica descritos para una β-xilosidasa frente a XOS naturales de 3 a 6 unidades. Los estudios con alcoholes de alcanos revelaron que, como otras glicosidasas de la familia GH3, BxTW1 mostraba la capacidad de catalizar reacciones de transxilosilación. Un análisis más detallado amplió el rango de posibles aceptores de la enzima a alcoholes de azúcar, monosacáridos y disacáridos. Además de esta versatilidad, se comprobó que los rendimientos de la transxilosilación eran altos y que además el proceso tenía lugar de forma regioselectiva. La promiscuidad de aceptor junto con la regioselectividad y sus propiedades fisico-químicas y cinéticas sugerían que BxTW1 podía ser una herramienta biotecnológica de interés.

Expresión heteróloga de BxTW1 y síntesis de un xilósido con propiedades antitumorales

Con el fin de mejorar los rendimientos en la obtención de la β-xilosidasa de *T. amestolkiae* a partir de los cultivos fúngicos, se expresó el gen *bxtw1* en la levadura *Pichia pastoris*. La cepa recombinante secretó niveles de rBxTW1 10 veces mayores que los del hongo y al mismo tiempo se logró una purificación de la enzima mucho más sencilla, en un único paso cromatográfico. La disponibilidad de altas cantidades de enzima pura permitió continuar con la caracterización del potencial de transxilosilación de rBxTW1, evaluando una librería de potenciales aceptores de muy distinta naturaleza. El resultado del ensayo mostró que esta glicosidasa puede utilizar como aceptores diversos derivados aromáticos, como los naftoles. Esto llevó a la síntesis de 2-(6-hidroxinaftil) β-D-xilopiranósido, un compuesto descrito como antiproliferativo selectivo, a partir de 2,6-dihidroxinaftaleno y xilobiosa. La optimización del proceso aplicando un método de respuesta en superficie (el diseño Box-Behnken) aumentó unas 8 veces el rendimiento en la producción del xilósido respecto a las condiciones inicialmente probadas.

Síntesis de un derivado xilosilado de hidroxitirosol altamente neuroprotector

La capacidad de rBxTW1 para xilosilar distintos compuestos aromáticos motivó la búsqueda de nuevos aceptores de transxilosilación con importancia industrial. En este caso se seleccionaron antioxidantes fenólicos de origen vegetal debido al interés existente en la obtención de sus derivados glicosilados. De entre los aceptores ensayados, se obtuvieron xilósidos de catecol, hidroquinona e hidroxitirosol, siendo este último el que despertó mayor interés, tanto por su novedad como por el alto rendimiento obtenido en su transxilosilación.

El hidroxitirosol es el antioxidante más potente presente en el aceite de oliva y el estudio detallado de su glicosilación demostró la síntesis

regioselectiva del xilósido 3,4-dihidroxifenil-etil-*O*-β-D-xilopiranósido. Los niveles de producción alcanzados fueron los más altos descritos hasta la fecha para la obtención enzimática de cualquier derivado glicosilado de este antioxidante. Mediante aproximaciones *in vitro*, utilizando cultivos celulares, se demostró que el nuevo compuesto mostraba propiedades antiinflamatorias similares a las del hidroxitirosol, pero su capacidad neuroprotectora era muy superior, en las condiciones en las que se realizaron estos ensayos.

Utilización de una endoxilanasa de T. amestolkiae *para la producción enzimática de xilooligosacáridos*

Se purificó y caracterizó una nueva endoxilanasa de los cultivos *T. amestolkiae*. A nivel bioquímico, la enzima purificada (XynM) mostró alta selectividad y una masa molecular pequeña (~20 kDa), propiedades típicamente asociadas a la familia GH11. Los perfiles de actividad en relación a pH y temperatura son similares a los de otras xilanasas descritas de *Penicillium* y *Talaromyces*, aunque destacando por su tolerancia al Cu^{2+} y al Pb^{2+}. Una vez caracterizada, esta endoxilanasa fue utilizada para la producción de XOS a partir de xilano de abedul. La caracterización de la mezcla oligosacarídica obtenida reveló la presencia tanto de XOS neutros como cargados, siendo xilobiosa, xilotriosa y xilotetraosa los productos mayoritarios. Por otra parte, la mezcla destacó por su contenido despreciable en xilosa, un monosacárido sin valor nutracéutico. Finalmente, su potencial prebiótico fue evaluado mediante la fermentación de heces de bebé lactante, analizándose el perfil de ácidos orgánicos en los cultivos y el microbioma bacteriano resultante. Ambos ensayos confirmaron el carácter prebiótico de estos XOS, que poseían capacidades bifidogénicas y destacaron por promover fuertemente el crecimiento de *Staphylococcus hominis*, un organismo considerado en estudios recientes como potencial probiótico.

Conclusiones

Como resultado de los estudios llevados a cabo en la presente tesis doctoral se han aislado y caracterizado dos enzimas xilanolíticas que pueden ser aplicadas en la obtención de productos de valor añadido a partir del xilano. La β-xilosidasa mostró un amplio rango de posibles aceptores de transxilosilación, acompañado de altos rendimientos y regioselectividad. El aprovechamiento de estas características permitió la síntesis enzimática de dos xilósidos con propiedades bioactivas: el agente antiproliferativo selectivo 2-(6-hidroxinaftil) β-D-xilopiranósido y el antioxidante neuroprotector 3,4-dihidroxifenil-etil-*O*-β-D-xilopiranósido. Respecto a la endoxilanasa, tras su caracterización se ha demostrado que puede ser

utilizada para la obtención de una mezcla de xilooligosacáridos, a partir de xilano de abedul, con notables capacidades prebióticas. Este trabajo abre nuevas posibilidades para la obtención de xilósidos y derivados de xilano de interés y pone de relieve el papel de los hongos filamentosos como excelentes fuentes de enzimas para la valorización de la biomasa lignocelulósica.

SUMMARY

Introduction

Xylan represents the second carbon reservoir in the biosphere, only preceded by cellulose. It is a heteropolysaccharide belonging to the group of hemicelluloses, therefore it is a part of most of the main sources of lignocellulosic biomass. Structurally, it is composed by a backbone of β-1,4-linked D-xylopyranosyl units, which is frequently acetylated and highly branched by short side chains of arabinose or glucuronic acid. The abundance of each of these substituents depends largely on the nature of the plant biomass. Due to its complexity, xylan hydrolysis requires the concerted action of multiple enzymes, among which two types of glycosidases, endo-β-1,4-xylanases and β-xylosidases, play the major roles. The former hydrolyze the polysaccharide by attacking internal links in the main chain, releasing oligosaccharides with different polymerization degrees. β-xylosidases end the process by converting these xylooligosaccharides (XOS) into xylose.

The economical relevance of exploiting this heteropolysaccharide is based on its great abundance and has driven the research on both xylanolytic enzymes. Industry is interested both in xylan saccharification for obtaining biofuels, and in its conversion into high value-added products. Attending to the latter possibility, endoxylanases can be applied for producing XOS, which are currently considered emerging prebiotics. Regarding to β-xylosidases, many of these glycosidases display the capacity of transferring a xylosyl residue to an acceptor compound, in a reaction called transxylosylation. By this way bioactive glycosides could be obtained, opening a new field for the application of these catalysts.

The xylanolytic enzymes are produced in nature mainly by bacteria and fungi for degradation of plant cell wall polysaccharides. Among these organisms, filamentous fungi are the ones which have aroused the greatest interest as producers of these enzymes. The reasons are the higher levels of xylanolytic activities displayed by fungi and the frequent secretion of the desired enzymes to the extracellular medium, which facilitates both the purification and direct use of fungal crudes for several applications.

Aims

Talaromyces amestolkiae CIB is an ascomycete fungus isolated from cereal wastes and identified in the laboratory of Professor María Jesús Martínez, in the Centre of Biological Research, where this doctoral thesis has been carried out. The cellulolytic system of this fungus had been

previously characterized, displaying very interesting properties. For this reason, the studies on this organism continued with the analysis of the enzymes involved in xylan degradation. With this purpose, the following tasks were established:

a) Study of the production by *T. amestolkiae* of enzymes implicated in xylan degradation.

b) Purification and characterization, from a biochemical and molecular perspective, of the main xylanolytic enzymes secreted by this fungus.

c) Study of the potential biotechnological applications of these biocatalysts for the valorization of plant biomass.

Results

Study of the production of xylanolytic enzymes by T. amestolkiae

The levels of xylanolytic activity reached by *T. amestolkiae* were assayed by culturing the fungus in liquid media in the presence of different carbon sources. Among the potential inducers tested, beechwood xylan in a concentration of 2% (w/v) was the one which led to the maximal secretion of both β-xylosidase and endoxylanase activities. These conditions were kept for the production of the enzymes of interest in the rest of this work.

Characterization of a novel β-xylosidase of biotechnological interest

A novel β-xylosidase was purified and characterized from *T. amestolkiae* cultures. Further sequencing of its gene led to its classification into the glycosyl hydrolase family 3. The enzyme showed about 10% *N*-glycosylation and a dimeric quaternary structure. Its characterization revealed several properties of interest. At the physicochemical level, the enzyme displayed a remarkable tolerance to the presence of Cu^{2+}, which is a strong inhibitor of most of the known xylanolytic and cellulolytic enzymes. Its maximal activity occurred at highly acidic values (pH 3), although it displayed high stability in a wide pH range (2-9). In terms of kinetics, it is particularly remarkable that the enzyme showed the greatest values of catalytic efficiency reported for a β-xylosidase against natural XOS from 3 to 6 units. Studies using alkan-ols revealed that, as described for other GH3 glycosidases, BxTW1 demonstrated the capacity of catalyzing transxylosylation reactions. An in-depth analysis extended its potential acceptor range to sugar alcohols, monosaccharides and disaccharides. In addition to this versatility, it was established that transxylosylation yields were high and the process took place in a

regioselective way. Its acceptor promiscuity, regioselectivity and physico-chemical and kinetic properties suggest that BxTW1 may be a biotechnologically interesting tool.

Heterologous expression of BxTW1 and synthesis of a xyloside with antitumor properties

With the purpose of improving the yields of β-xylosidase from the fungal cultures of *T. amestolkiae*, the gene *bxtw1* was expressed in the yeast *Pichia pastoris*. The recombinant strain secreted rBxTW1 levels surpassing by 10-fold those released by the native producer. In addition, enzyme purification became much simpler and was accomplished in a single chromatographic step. The availability of high quantities of purified enzyme allowed progressing on the characterization of the transxylosylation potential of rBxTW1 using a compound library containing potential acceptors with very different natures. The result of the assay showed that this glycosidase can use several aromatic derivatives, including naphthols, as acceptors. This led to the synthesis of 2-(6-hydroxinaphtyl) β-D-xylopyranoside, a compound reported as selective antiproliferative, from 2,6-dihydroxynaphtalene and xylobiose. The optimization of the process by applying the Box-Behnken design, a response surface methodology (RSM), increased the production of the xyloside about 8-fold when compared to the initial conditions.

Synthesis of a highly neuroprotective xylosyl derivative from hydroxytyrosol

The capacity of rBxTW1 for xylosylating several aromatic compounds led to the search of novel transxylosylation acceptors of industrial interest. In this case, some plant phenolic antioxidants were selected attending to the existing interest in obtaining their glycosyl derivatives. Among the tested acceptors, xylosides from catechol, hydroquinone and hydroxytyrosol were obtained, being the latter especially interesting due both to its novelty and high transxylosylation yield.

Hydroxytyrosol is the most potent antioxidant in olive oil and the in-depth study of its enzymatic glycosylation demonstrated the regioselective synthesis of the xyloside 3,4-dihidroxyphenyl-ethyl-*O*-β-D-xylopyranoside, The production levels achieved were the highest reported up to date for the enzymatic synthesis of glycosyl derivatives from this antioxidant. *In vitro* assays using cell cultures demonstrated that the novel compound displayed similar anti-inflammatory properties as the aglycon, but its neuroprotective capacity was remarkably superior in the assayed conditions.

Enzymatic production of xylooligosaccharides by an endoxylanase from T. amestolkiae

A novel endoxylanase was purified and characterized from the crudes of *T. amestolkiae*. At the biochemical level, the purified enzyme (XynM) displayed high selectivity and low molecular mass (~20 kDa), which are properties typically associated with the GH11 family. The activity profiles related to temperature and pH were similar to other reported xylanases from *Penicillium* and *Talaromyces*, although its tolerance to Cu^{2+} and Pb^{2+} must be highlighted. Once characterized, this endoxylanase was applied to the production of XOS from birchwood xylan. The characterization of the oligosaccharide mixture obtained revealed the presence of both neutral and charged XOS, being xylobiose, xylotriose and xylotetraose the main products. In addition, the negligible content of xylose in the mixture is remarkably positive, given the fact that this monosaccharide has no nutraceutic value. Finally, its prebiotic potential was evaluated by fermentation of feces from a breast-fed child, determining the profile of organic acids in the cultures and the bacterial microbiome developed. Both assays confirmed the prebiotic properties of these XOS, which demonstrated bifidogenic capacity, outstanding also for strongly promoting the growth of *Staphylococcus hominis*, an organism considered as a potential prebiotic in recent studies.

Conclusions

The studies developed in this doctoral thesis have led to the isolation and characterization of two xylanolytic enzymes that can be applied to the obtention of added-value products from xylan. The β-xylosidase displayed a wide range of potential transxylosylation acceptors together with high reaction yields and regioselectivity. The exploitation of these features allowed the enzymatic synthesis of two xylosides, whose bioactive properties have been demonstrated: 2-(6-hydroxynaphtyl) β-D-xylopyranoside, a selective antiproliferative agent, and the neuroprotective antioxidant 3,4-dihidroxiphenyl-ethyl-*O*-β-D-xylopyranoside. Regarding to the endoxylanase, it was characterized and subsequently used for obtaining a xylooligosaccharides mixture from birchwood xylan, with remarkable prebiotic capacities. This work opens new possibilities for the enzymatic production of xylosides and xylan derivatives of interest, and highlights the role of filamentous fungi as valuable sources of enzymes for lignocellulosic biomass valorization.

1. INTRODUCTION

1.1. CARBOHYDRATES

According to the well-known Lehninger's Principles of Biochemistry, carbohydrate is a general denomination for polyhydroxy aldehydes, polyhydroxy ketones and other molecules whose hydrolysis yields these compounds. The composition of most carbohydrates corresponds to the empirical formula $C_nH_{2n}O_n$, although these ratios are frequently altered due to the presence of functional groups as acetyl, methyl or other including different chemical species, usually nitrogen, phosphorus or sulfur (Nelson and Cox, 2008).

The ethymology of the term carbohydrate originates in the first hypothesis about sugars, which considered these substances to be composed by carbon and water, namely carbon-hydrates. Although further studies demonstrated that they were actually polyhydroxylated organic compounds, the name carbohydrate, together with "saccharide" and "sugar" are still in use (Kamerling, 2007; National Research Council, 2012).

The simplest carbohydrates, constituted by a single unit of polyhydroxy aldehyde or ketone, are called monosaccharides and contain three or more carbon atoms. These basic blocks can form more complex saccharides when they combine with each other through *O*-glycosidic bonds. The glycidic chains containing two to ten monosaccharidic residues and are named oligosaccharides and, if longer, they are considered polysaccharides. All monosaccharides have reducing power, as their carbonyl carbon, also known as anomeric carbon, is free and therefore it can be oxidized. More complex sugars may have reducing power or not, depending on the existence of a free anomeric carbon in the molecule. For instance, in the maltose disaccharide (α-D-glucopyranosyl-(1→4)-α-D-glucopyranose) one of the two residues has a free anomeric carbon and hence, has reducing power whereas sucrose is a non-reducing disaccharide (α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) because both carbonyls participate in the linkage (Nelson and Cox, 2008). In addition of being essential for understanding the reactivity of carbohydrates, reducing power is on the basis of many classical methods for assessing the hydrolysis of polysaccharides (Miller, 1959; Nelson, 1944). At least one anomeric carbon is involved in a glycosidic bond; therefore the hydrolysis of an oligo- or polysaccharide will always lead to a product mixture with increased reducing power, as it is shown in Figure 1.1.

Carbohydrates composed of a glucidic part attached to a non-saccharide compound (proteins, lipids, nucleic acids, polyphenols…) are called glycosides or glycoconjugates.

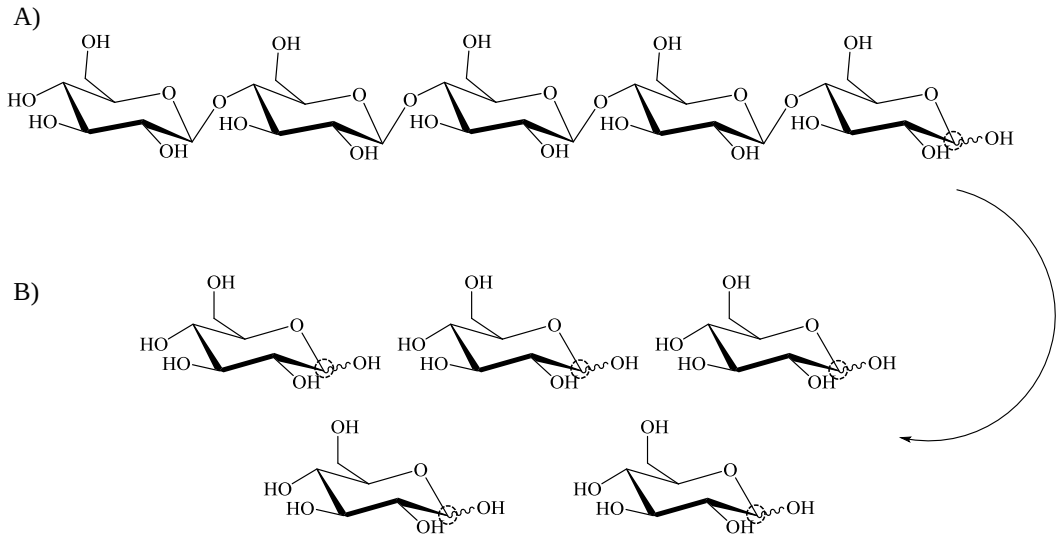


Fig. 1.1. Complete hydrolysis of cellopentaose (A) in D-glucose (B). The free anomeric carbons are marked with dashed circles to highlight the increase of reducing units after hydrolysis.

As mentioned before, carbohydrates larger than monosaccharides come from the linkage of additional sugar units by glycosidic bonds, generally of the *O*-type. These covalent bonds are acetals/ketals formed by reaction of the anomeric carbon of a carbohydrate (hemiacetal/hemiketal) with any of the free hydroxyl groups of another (Nelson and Cox, 2008; Bochkov and Zaikov, 1979), releasing a molecule of water. They are extremely stable linkages, whose spontaneous disruption has an estimated half life of several million years (Wolfenden et al., 1998), although they are susceptible to acid hydrolysis (Adams, 1965). Besides *O*-glycosidic bonds (Fig. 1.2A), carbohydrates with *N*-linkages (Fig. 1.2B) are frequently found in nature, being glycoproteins (Roth, 2002) and nucleotides the main examples (Nelson and Cox, 2008). Finally, *C*- (Fig. 1.2C) (Nicotra, 1997) and *S*-glycosides (Fig. 1.2D) (Jahn et al., 2003) are rare in nature (Fahey et al., 2001; Xie et al., 2003) and are obtained mainly by chemical or enzymatic synthesis. As in conventional *O*-linkages, a hemiacetal participates in the covalent bond, but the other component is, respectively, carbon or sulfur. Figure 1.2 displays an example of each type of glycoside.

The importance of carbohydrates has led to the development of a specific discipline for its study, known as glycoscience.

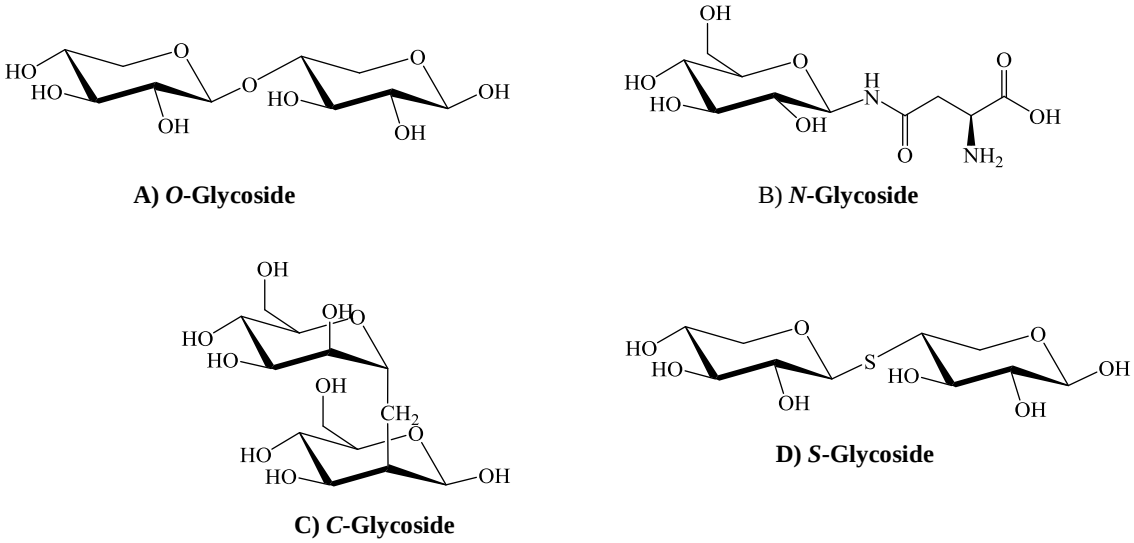


Fig. 1.2. Main types of glycosides. Xylobiose, an example of *O*-glycoside (A), β-*N*-glycosyl-L-asparagine, an example of *N*-glycoside (B), α-1,2-*C*-mannobioside, an example of *C*-glycoside (Espinosa et al., 1999) (C), 4-thioxylobiose, an example of *S*-glycoside (Defaye et al., 1985) (D).

1.2. GLYCOSCIENCE AND CARBOHYDRATE ACTIVE ENZYMES

Carbohydrates are, together with lipids, proteins and nucleic acids, essential components of the biosphere. Indeed, these biomolecules represent more than 50% of the total weight of biomass on Earth. They are universally distributed and constitute a necessary part of every life form. Sugars have multiple natural functions, although they can be summarized in three: 1) energetic, as the main reservoir and substrate for cellular metabolism; 2) structural, as components of the cell walls and the extracellular matrix; 3) cell communication and signaling, as sites of recognition (Kamerling, 2007; National Research Council, 2012). These properties have boosted carbohydrates to play a central role in an increasing number of applications, comprising fields as diverse as health (Comstock and Kasper, 2006; Dube and Bertozzi, 2005; Macfarlane et al., 2006), fuels (Durre, 2007; Gray et al., 2006) or materials science (DiGregorio, 2009; Pashkuleva and Reis, 2010). At the same time, saccharides are studied by a vast number of disciplines, as it is the case of biochemistry, cell biology, chemical biology, medicine, pharmacology or biotechnology. Because of this, glycoscience has been defined as an interdisciplinary field aimed to a better understanding of the structures, functions and uses of carbohydrates (National Research Council, 2012).

The study of the enzymes that catalyze the synthesis, disruption or modification of sugars is one of the main interests of glycoscience. These biocatalysts receive the name of carbohydrate active enzymes or CAZymes

and most of them are included and classified in CAZy (Carbohydrate Active Enzymes database, www.cazy.org/), a public database where the enzymes are grouped according to their protein sequence similarity (Lombard et al., 2014).

Currently, CAZy contains information about five large enzyme classes: 1) glycosyl hydrolases (GHs), which catalyze the cleavage of a glycosidic bond releasing a sugar hemiacetal (Davies and Henrissat, 1995); 2) glycosyltransferases (GTs), catalysts of the synthesis of carbohydrates from nucleotide-sugars (Lairson et al., 2008); 3) polysaccharide lyases (PLs), which cut polysaccharides containing uronic acids through a β-elimination mechanism (Lombard et al., 2010); 4) carbohydrate esterases (CEs) which catalyze the cleavage of sugar esters (Biely et al., 1997); and 5) enzymes displaying auxiliary activities (AAs), which are basically oxidoreductases acting together with the former classes of CAZymes (Levasseur et al., 2013).

Glycosyl hydrolases, also known as glycosidases and glycoside hydrolases, was the first class in which families were defined and it is probably the most important group among CAZymes, not only because it has the largest number of deposited sequences, but also due to its wide variety of substrates (Herscovics, 1999a; Yamamoto et al., 2000), properties (Kengen et al., 1993; Mao et al., 2010) and applications (Liu et al., 2007; Rempel and Withers, 2008).

1.3. GLYCOSYL HYDROLASES

Even though glycosidic linkages are among the most stable in nature, their GH-catalyzed disruption is around 10^{17} times faster than their spontaneous hydrolysis. For this reason glycosidases are considered one of the most efficient catalysts available (Rye and Withers, 2000). As enzymes capable of hydrolyzing glycosidic bonds (*O*- and in a minor extent *S*-), they receive the identifier EC 3.2.1.- in the classification proposed by the NC-IUBMB (Nomenclature Committee of the International Union of Biochemistry and Molecular Biology) which groups enzymes based on the type of reaction catalyzed. The huge diversity of natural carbohydrates displays its counterpart in the high number of GH-type activities that have been defined as more glycosidases are analyzed. Thus, EC numbers assigned to glycosidases (http://enzyme.expasy.org/EC/3.2.1.-), comprise currently from EC 3.2.1.1 (α-amilase) to EC 3.2.1.196 (α-1,6-maltotetraose-hydrolase). At the same time the protein sequence-based CAZy classification displays currently a total of 135 GH families. When these criteria are considered simultaneously, it frequently occurs that a single CAZy family comprises enzymes with different EC number and that

enzymes from different families display the same EC. Nevertheless, in spite of this diversity, studies on glycosyl hydrolases portrait several general characteristics that allow accomplishing their overall description. These features, related to their catalytic mechanism and structure, are discussed below.

1.3.1. General features

Globally, glycosidases differ from each other attending to two criteria. Based on the position where the GH acts in the substrate two categories can be distinguished: 1) endoglycosidases, that cut linkages between internal residues of the glucidic chain, and 2) exoglycosidases, that cleave the glycosidic linkage at terminal positions of the chain, usually releasing mono- or disaccharides. The second criterion of classification is based on considering the anomeric configuration of the reaction product, and divide GHs into: 1) retaining glycosidases, which release a product whose anomeric configuration is the same as the initial substrate, and 2) inverting glycosidases, which operate generating products with the opposite configuration to the substrate in the anomeric carbon (Bojarova and Kren, 2009). The latter classification involves two different catalytic mechanisms proposed for the first time by Koshland (1953) and summarized in figure 1.3.

Inverting glycosidases operate through two acidic amino acids (Asp or Glu), one of them acting as a general base catalyst (carboxylate anion in the side chain) and the other one as a general acid catalyst (protonated carboxylic acid in the side chain). When a nucleophile, usually water, gets into the active site, it is deprotonated by the carboxylate (basic catalysis), allowing it to attack the anomeric carbon. This nucleophilic attack is assisted by the acid catalyst which transfers its proton to the released aglycon. It is, therefore, a single step mechanism in which the initially deprotonated catalyst gets a proton and *vice versa*. At the same time a saccharide product is released, whose anomeric configuration is opposite to that of the initial substrate (Fig. 1.3A).

Retaining enzymes also require the participation of two catalytic carboxyl groups (Asp or Glu), but catalysis occurs through two well-separated steps and, because of that, it also receives the name of double-displacement mechanism. In the first step, carboxylate acts as a nucleophile, attacking the anomeric center of the substrate with the catalytic assistance of the residue called catalytic acid/base, which donates a proton to the released aglycon. Thus, the first step ends with the formation of an enzyme-substrate intermediate. In the subsequent phase, an external nucleophile, as water, performs a second nucleophilic attack on the anomeric carbon of the substrate, disrupting the former intermediate. This new attack is also assisted by the second carboxylic residue, this time

acting as a general base catalyst, receiving a proton from the nucleophile in order to activate it. According to this mechanism, the released hemiacetal keeps the anomeric configuration of the initial carbohydrate (Fig. 1.3B) (Rempel and Withers, 2008).

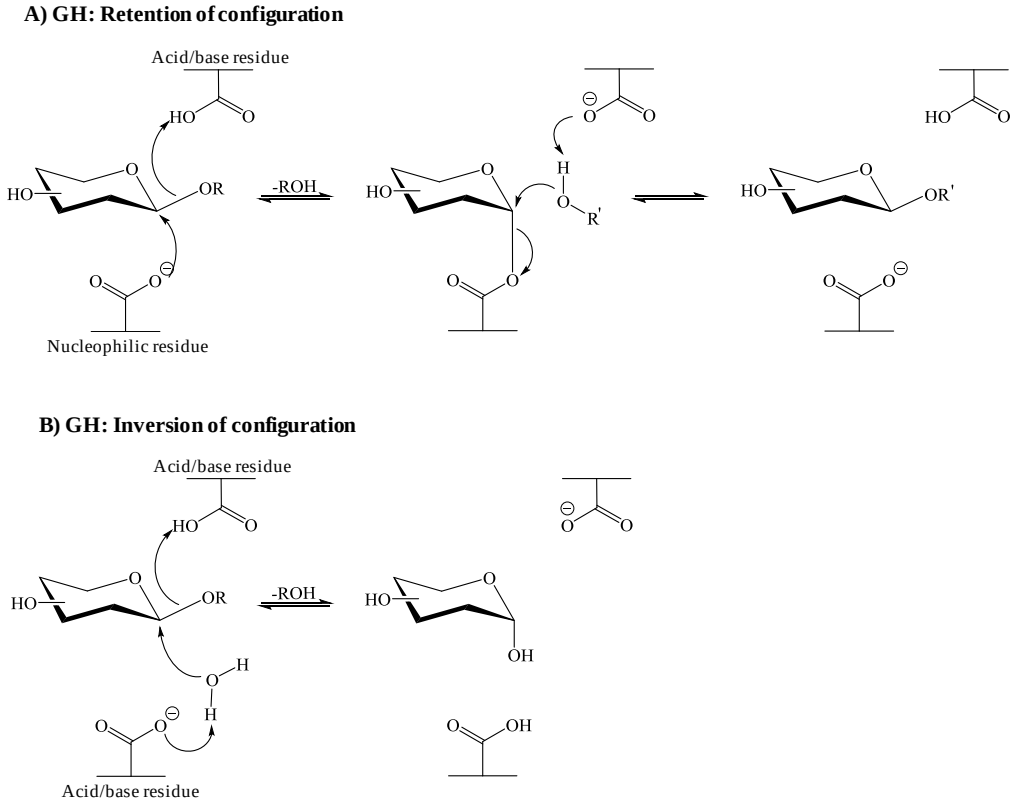


Fig. 1.3. Mechanisms of retaining (A) and inverting glycosidases (B)

Despite the fact that most of known GHs follow one of these two mechanisms, other alternatives have been found over time. Some of them involve the participation of a carboxyl group of the sugar substrate itself (Williams et al., 2002), a nucleophilic attack carried out by a residue different from Glu or Asp (Watts et al., 2003) or even the cleavage of the glycosydic bond trough a redox mechanism (Yip and Withers, 2006).

Another way to analyze the types of glycosidases is based on considering the class of reaction that they catalyze. According to this criterion, in addition to strictly hydrolytic GHs, there are others that have the ability to carry out also transglycosylation and/or reverse hydrolysis. Up to now we have mainly focused on the hydrolytic activity of GHs, as it is the most common for the vast majority of characterized glycosidases, but the other ways of catalysis display a great relevance from the perspective of synthetic processes. Due to its importance and interest for this doctoral thesis, transglycosylation will be analyzed in more detail in the following section. However, it should be mentioned that this type of reactions originates a new bond between a carbohydrate and an acceptor compound.

This process operates under kinetic control and it is necessarily preceded by the cleavage of another glycosidic linkage. The formation of a new bond also occurs in reverse hydrolysis, but, unlike the former case, the glycosidic linkage is formed *de novo*, without requiring the disruption of a previous bond. As the name suggests, this activity consists on the reverse reaction of hydrolysis and requires high substrate concentration (generally a monosaccharide) and a low-water reaction medium, substituting most of the aqueous environment by an organic solvent or an ionic liquid. It is a thermodynamically controlled process which it is usually associated to low yields and poor selectivity, limiting its interest and applications (Crout and Vic, 1998; Yang et al., 2012).

Regardless of the catalysis type and its mechanism, glycosidases share a basic structural feature, which is the presence of subsites. These are defined as contiguous regions inside the enzyme, formed by a series of residues interacting in a non-covalent way with the polymeric substrate units, in order to facilitate the structural and electrostatic recognition. In the active site of glycosyl hydrolases subsites are indicated numerically from the hydrolyzed glycosidic bond, taking into account the position of the non-reducing and reducing ends of the carbohydrate. Thus, subsites are defined based on the substrate unit that they accommodate, being named as (+1), (+2), (+3)… towards the end with reducing power and as (-1), (-2), (-3)… in the opposite direction. Therefore, hydrolysis always occurs between (+1) and (-1) subsites (Päes et al., 2008).

The complex catalytic machinery of GHs faces severe barriers to accomplish the efficient degradation of insoluble polysaccharides, given the presence of glycosidic bonds inaccessible to the enzymatic action. In order to overcome this hindrance, many glycosidases have adopted a modular conformation in which the catalytic module, containing the active site, can be attached to one or several non-catalytic units, known as carbohydrate binding modules (CBMs). Currently, these protein regions are considered to play from one to three different roles: 1) Keeping the enzyme close to the substrate, increasing the concentration of glycosidases in the carbohydrate surroundings and, by this way, favoring its degradation; 2) Directing the enzyme to the substrate's specific region in which it is going to exert its catalytic action, as for example the non-reducing end of a cellodextrin; and 3) Disrupting the substrate, as it occurs for some cellulases whose CBMs decrease the crystallinity of cellulose in the regions of the interaction. These binding domains can adopt a wide variety of structures and have their own classification in the CAZy database, which today comprises 81 families (Boraston et al., 2004; www.cazy.org).

1.3.2. Transglycosylation

As it has been introduced in the previous section, the classical vision about glycosidases is referred to their hydrolytic capacities (the definition itself involves the disruption of a glycosidic bond). However, the ability of these enzymes in synthesizing this type of linkage is known for over 60 years and the main activity in this sense is called transglycosylation (Edelman, 1956). In spite of being, apparently, a function opposite to regular catalysis, it follows the same mechanism displayed by retaining glycosidases and it is an exclusive ability of this type of GHs (Kitaoka, 2010). Thus, the reaction is denominated hydrolysis when the second nucleophilic attack is carried out by water, but it is considered transglycosylation if this external agent is any other compound. In the latter case, the result of the reaction is no other than the transfer of a sugar unit from a donor substrate to a nucleophilic acceptor, with the formation of a new glycosidic bond. Therefore, transglycosylation is still comprised into the proposed definition for glycosyl hydrolases, because the first step of double displacement involves the disruption of a linkage and the formation of a carbohydrate hemiacetal (Crout and Vic, 1998; Bissaro et al., 2015).

Glycosidases that preferentially catalyze the transfer of sugars to certain acceptors different from water can receive the name of transglycosylases. In this group there are examples of strict transglycosylation, but generally these enzymes can also catalyze hydrolytic reactions. Indeed, the difference between glycosidases and transglycosylases is not clear in many cases and the behavior of the enzyme in its natural environment is unknown (Bissaro et al., 2015). Among the more than 9,000 characterized GHs deposited in CAZy, just a few have demonstrated transglycosylation activity (Bissaro et al., 2015; Lombard et al., 2014) and although this type of enzymes has been typically associated to exoglycosidases, currently there is an increasing number of reports about endo-transglycosylases (Yamamoto et al., 1994; Eneyskaya et al., 2003; Fry et al., 1992; Kono et al., 1999; Madhuprakash et al., 2012).

The ability of GHs for catalyzing transglycosylation reactions has converted these enzymes into an alternative to chemical approaches for obtaining oligosaccharides and glycoconjugates. Chemical synthesis usually involves the formation of toxic byproducts and display low regio- and stereoselectivity, therefore it requires a high number of protection and deprotection steps (Danby and Withers, 2016). On the contrary, glycosidases show a complete stereoselectivity and a remarkably greater regioselectivity, although it is not generally complete.

Together with glycosidases, glycosyltransferases represent the other major alternative to chemical approaches. As an advantage over most GHs, GTs are more regioselective, but they require the use of expensive nucleotide-sugars as donors, while glycosidases can catalyze carbohydrate

transfer from low cost sugars like polysaccharides derived from biomass (Hancock et al., 2006; Nilsson, 1991; Weignerova et al., 2009).

Despite the mentioned advantages, GHs-catalyzed glycosylation also displays some weaknesses. Major limitations are: 1) the low production yield of the new glycoside or oligosaccharide and 2) the need for taking a careful kinetic control of the reaction that is always reversible, which can lead to hydrolysis of the desired product (Jahn and Withers, 2003). Some approaches devoted to overcome these drawbacks have been reported, mainly comprising the decrease of the water content in the reaction and enzyme mutation.

The idea of diminishing water concentration attempts to minimize the competence between hydrolysis and transglycosylation for the donor and, at the same time, making more difficult the hydrolysis of the glycosylated product. For that, catalysis is generally performed in organic environments, which has the limitation of the low stability of GHs in these media, complicating the process (Mori et al., 1997). This drawback can be solved by using ionic liquids (ILs), providing by this way a polar environment without the presence of water (Gorke et al., 2010), although the spread of this alternative has been slowed down because of the high cost of ILs.

Currently, the most successful solutions seem to be the ones relying on the direct action on the enzyme, instead of the reaction medium. This approach has led to obtaining two major mutant classes, glycosynthases (Mackenzie et al., 1998) and thioglycoligases (Jahn et al., 2003). Both variants are based on the replacement of any of the catalytic amino-acids by an inert one. These approaches were initially developed on glycosidases displaying double displacement mechanism, but they have already been adapted to inverting GHs (Honda and Kitaoka, 2006; Honda et al., 2008). In the case of glycosynthases, the replaced residue is the one responsible of the nucleophilic attack; hence the mutant is not able to catalyze the first step of the double displacement. Because of this, glycosynthases require the addition of an activated donor, mimicking the enzyme-substrate intermediate. This intermediate is conventionally attacked by the nucleophilic acceptor (Fig. 1.4A) and by this way a non-hydrolysable product is obtained. Regarding to thioglycoligases, they are obtained by replacing the catalytic acid/base. Without its assistance, mutants need a donor with a good leaving group, like dinitrophenyl sugars, to succeed in the formation of the enzyme-substrate intermediate and, then, a strong nucleophile in order to disrupt it. Thioglycoligases offer an effective method for synthesizing *S*-glycosidic linkages instead of *O*-glycosidic, because of its capacity for transferring a carbohydrate to thiosugars (strong nucleophiles) (Fig. 1.4B). The obtained *S*-glycosides are inhibitors of wild-

type GHs, displaying a wide variety of applications (Hancock et al., 2006; Jahn and Withers, 2003).

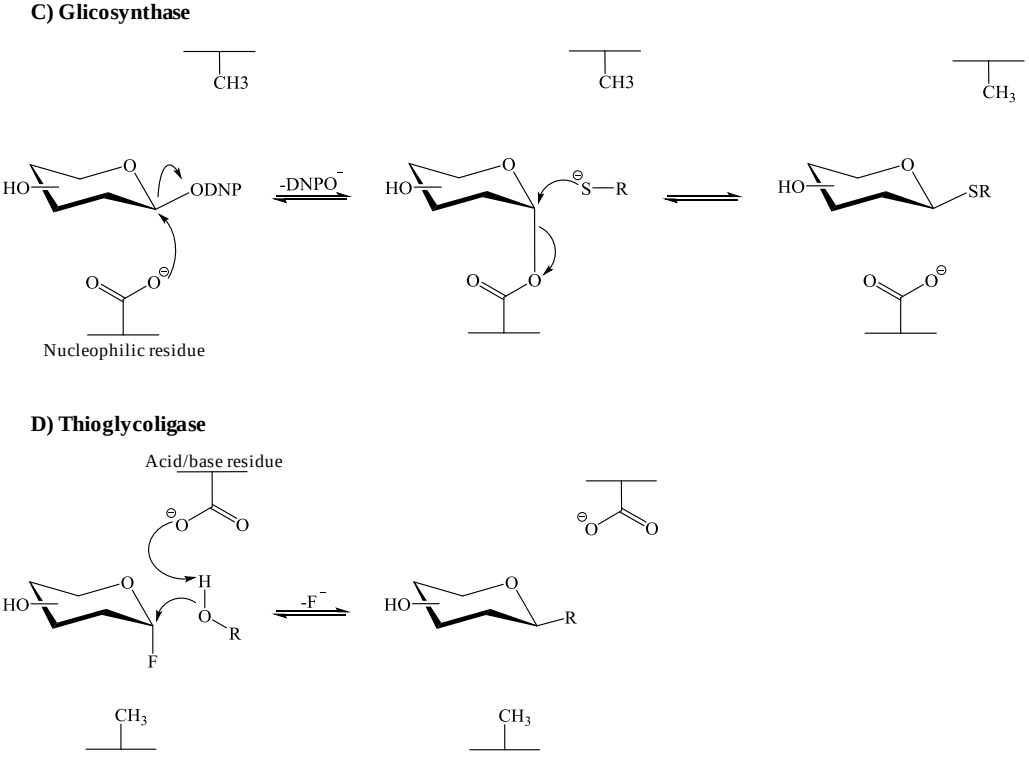


Fig. 1.4. Mechanisms of the main glycosidase mutants: glycosynthases (A) and thioglycoligases (B).

1.3.3. Applications of glycosyl hydrolases applications

Taking into account the variety of substrates for GHs, their catalytic mechanisms and the great number of reported enzymes, it is not surprising that they are one the most produced enzymatic catalysts at the industrial level over the world, only preceded by proteases and lipases, with plenty of proposed applications. Among their possible uses, there are promising ideas, which are still under research, together with others commercially exploited for decades. The current market field of glycosidases comprises, at least, the food, textile and energy industries (Polaina and MacCabe, 2007). Some of the most remarkable applications in which wild-type glycosyl hydrolases play a central role are summarized below (the use of glycosynthases and thioglycoligases is still quite limited).

-Clinical applications: The medical field constitutes a particular case because the importance of glycosidases does not rely on their catalytic properties (the most conventional approach), but on the attempt of inhibiting or restoring that activity. The essential function developed by some GHs in pathogenesis, either by virus (Homman-Loudiyi et al., 2003; Suzuki et al., 2005), bacteria (Marion et al., 2009; Prizont, 1982), protozoos (Jacobson et al., 2001; Jones et al., 2005) or fungi (Mora-Montes

et al., 2007; Fisher and Woods, 2000), as well as their importance in the development of cancer (Bernacki et al., 1985) or in diabetes (Scheen, 2003; Van de Laar et al., 2005) have converted the treatments with glycosidase inhibitors into an emerging field (Asano, 2003; Gerber-Lemaire and Juillerat-Jeanneret, 2006). The opposite case deals with diseases caused by the malfunction of an endogenous glycosidase, whose main examples are found in the group of the lysosomal diseases (Cox, 2012). The treatment of these disorders is always palliative and it is carried out mainly by enzymatic replacement, producing the required enzyme heterologously and providing it to the patient (Brady, 2006). Chaperones can also be used in order to recover the right folding of the altered endogenous enzyme, and consequently, its activity (Parenti, 2009).

-Enhancement of bioactive compounds. The capacity of GHs for catalyzing transglycosylation reactions makes them a biotechnological tool for the modification of several compounds of interest, through the addition of one or more units of carbohydrate. Several beneficial effects have been reported for the glycosides obtained by transglycosylation, among them their increased solubility (Kometani et al., 1994; Torres et al., 2011; Woo et al., 2012), biosafety (Prodanovic et al., 2005) and stability (Woo et al., 2012; Yamamoto et al., 1990). Sometimes, the addition of the sugar moiety does not change these properties, but determines the appearance of the bioactivity itself as it is the case of alkylglycosides (Kouptsova et al., 2001; Turner et al., 2007). On the other hand, GHs can be used for the opposite purpose, when the beneficial effect is associated to deglycosylation of the bioactive compound (Li et al., 2008; Pyo et al., 2005).

-Obtaining of the "stone-washed" appearance. Textile industry is the global major consumer of cellulases, specifically β-1,4-endoglucanases (EC 3.2.1.4), mainly for getting the "stone-washing" or "biostoning" effect of denim. The role of these enzymes in the process is to diminish the crystallinity of cellulose fibers. This gives regions where the dye can be easily removed, generating the desired aged look. The use of cellulase in this industry has replaced the traditional abrasion procedure with pumice, which is expensive and more difficult to control (Montazer and Maryan, 2010; Pazarlioglu et al., 2005).

-Juice clarification. Fruit juice commonly has a cloudy appearance mainly due to the presence of polysaccharides, among which pectin appears at high concentration. Pectinases and, to a minor extent, other glycosidases have emerged as solutions for this problem at the industrial level. These enzymes have been used in clarification treatments degrading the juices' polysaccharides into more soluble sugars decreasing turbidity, which leads to a more attractive product for the consumer (Sandri et al., 2011; Sreenath and Santhanam, 1992).

-Improvement of bakery products. The bakery industry has been using α-amilases (EC 3.2.1.1) for decades in order to enhance starch saccharification and, consequently, the yeast's fermentation yields. By this way, the volume increases and the texture of the final product improves. Recently, other enzymes have been added to the process, attempting to delay the phenomenon known as bread staling, which alters the firmness of the crumb and the water content, softening the crust and giving a worse taste. This alteration has an estimated economic cost of more than one billion dollars, just considering the United States. The addition of glycosidases as xylanases, cellulases or pullulanases (EC 3.2.1.41), besides other activities, significantly extends the time that bakery products are kept in optimum conditions (Gupta et al., 2003; Van der Maarel et al., 2002).

-Production of prebiotics. Prebiotics are defined as food ingredients, non-digestible by the host, that have a beneficial effect through their selective metabolism in the intestinal tract (Gibson et al., 2004). Most of the known prebiotics are obtained from controlled hydrolysis of polysaccharides, as it is the case of fructooligosaccharides (FOS) derived from inulin and xylooligosaccharides (XOS) derived from xylan, or by glycosylation, as the production of galactooligosaccharides (GOS) and fructooligosaccharides from lactose and sucrose respectively. Both approaches can be performed chemically or using GHs (Crittenden and Playne, 2008).

-Production of biofuels. The hydrolytic abilities of glycosidases make them the most important catalysts in the saccharification of plant biomass for production of second-generation bioethanol. In this process, plant cell wall polysaccharides are hydrolyzed into their corresponding monosaccharides, essentially glucose and xylose, which are then fermented in order to obtain ethanol. An efficient saccharification requires the concerted action of different types of glycosidases, mainly β-1,4-endoglucanases (EC 3.2.1.4), exo-cellobiohydrolases (EC 3.2.1.91), β-glucosidases (EC 3.2.1.21), endo-1,4-β-xylanases (EC 3.2.1.8), β-xylosidases (EC 3.2.1.37) and pectinases (EC 3.2.1.15) (Sweeney and Xu, 2012; Zhao et al., 2013).

Figure 1.5 displays a scheme summarizing and giving examples of many of these applications.

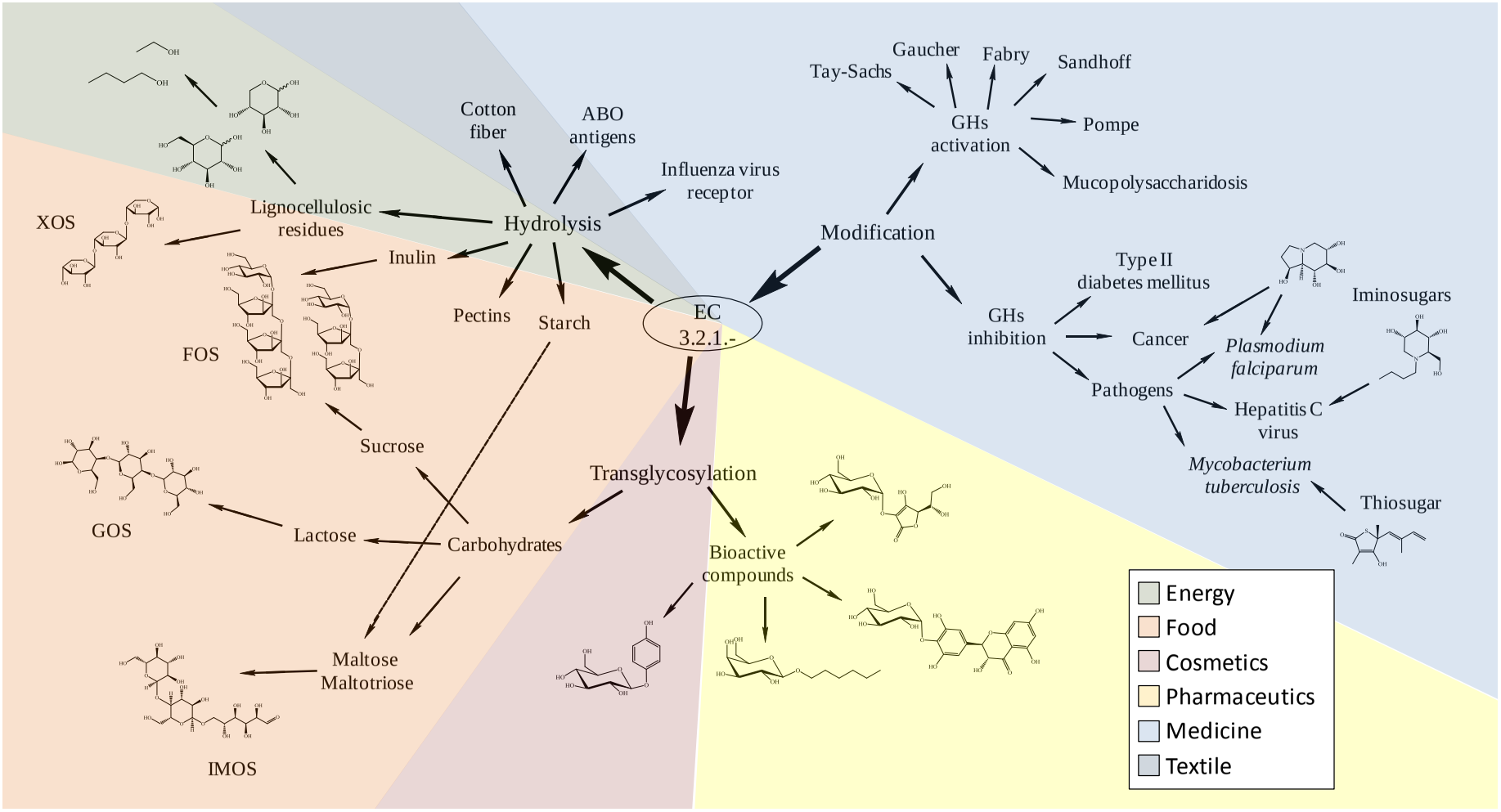


Fig. 1.5. Glycosyl hydrolases: properties, applications, examples and areas of interest.

1.4. LIGNOCELLULOSIC BIOMASS

As already mentioned, glycosyl hydrolases play a central role in the exploitation of lignocellulosic biomass, which is not only the most abundant carbohydrate material in nature, but also the main renewable resource of biosphere, with an annual production of about $2 \cdot 10^{11}$ tons (Zhang, 2008). Lignocellulose is the essential component of plant cell walls and it is defined as a dynamic and complex structure of cellulose microfibrils embedded in a matrix of hemicellulose and strongly reinforced by the presence of lignin. The latter is an amorphous heteropolymer, highly branched and composed by phenylpropane units (Pandey and Kim, 2011), which establishes a network of hydrophobic interactions with cellulose. The balance between these three components changes depending not only on the plant species, but also on the selected tissue and the stage of development of the organism (Taiz and Zeilinger, 2002). The content of these polymers according to the plant type is gathered in Table 1.1.

Table 1.1. Composition of different lignocellulosic materials (Betts et al., 1991)

Material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods	45-55	24-40	18-25
Softwoods	45-50	25-35	25-35
Grasses	25-40	25-50	10-30

The main properties of cellulose and hemicellulose, the most important polysaccharides in plant cell wall, will be shortly described below.

1.4.1. Cellulose: structure and characteristics

Cellulose is a homopolysaccharide composed of units of D-glucopyranose linked by β-1,4 *O*-glycosidic bonds, constituting long linear chains. Its degree of polymerization differs in the primary and secondary plant cell wall being higher in the latter, which contains 10,000 to 15,000 glucose residues. In both cases, groups of between 30 and 200 chains of β-1,4-glucopyranose are arranged in parallel and interact to each other through inter- and intra-chain hydrogen bonds. The resulting structure is called cellulose microfibril (Klemm et al., 1998; O'Sullivan, 1997).

These microfibrils are the basis of microcrystalline cellulose, also known as cellulose I (Fig. 1.6A), an insoluble and highly recalcitrant polymer. In nature, this highly ordered structure is combined with the so-called amorphous cellulose (Fig. 1.6B), which is defined as non-crystalline regions in the interfibrilar space. Amorphous cellulose represents a small

percentage in the secondary plant cell wall, but it may be the main cellulosic structure in the primary plant cell wall (Klemm et al., 1998; O'Sullivan, 1997).

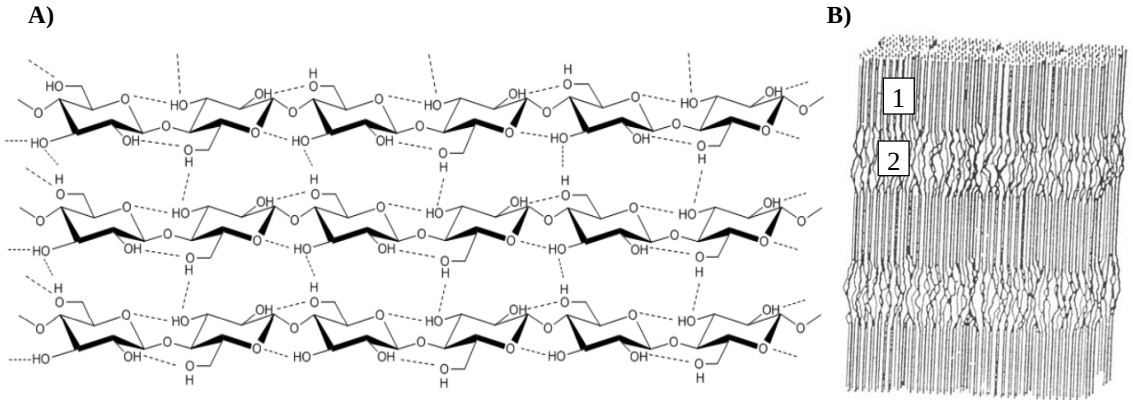


Fig. 1.6. Primary structure of cellulose I. The network of inter- and intra-molecular hydrogen bonds is shown. Adapted from Xi *et al*. (2013) (A). Cellulose microfibril: 1, crystalline region; 2, amorphous region. Adapted from Ioelovich (1999).

1.4.2. Hemicelluloses: types and distribution

The term hemicelluloses groups a series of polysaccharides with heterogeneous structures found in nature together with cellulose in the plant cell wall. Hemicelluloses are usually classified in four types: mannans, xyloglucans, mixed-linkage β-glucans and xylans, which differ from each other in the composition of their backbone and branches, and in the type and distribution of glycosidic linkages (Scheller and Ulvskov, 2010).

Mannans or galactomannans are partially branched polymers, in which the main chain is made up of β-(1,4)-linked mannopyranoses. Side chains of α-galactose are linked to the backbone through the *O*-6 position of mannose units (Fig. 1.7A). Glucomannans and glucogalactomannans can include β-1,4-glucopyranose residues in the main chain. This group of hemicelluloses is especially abundant in lycophytes and mosses (Scheller and Ulvskov, 2010; Ebringerova, 2006).

Xyloglucans display a cellulose-like backbone composed of β-(1,4)-linked glucopyranoses, but including side chains of α-xylopyranose attached to the *O*-6 positions of the main chain. Type I xyloglucans are associated to a branching pattern of three substituted glucoses followed by a non-branched one (Fig. 1.7B), while in type II xyloglucans two branched and two unsubstituted residues alternate along the chain. Regarding to their distribution, type I is usually found in primary cell wall of hardwoods and type II is frequent in *Solanaceae* (Scheller and Ulvskov, 2010; Ebringerova, 2006).

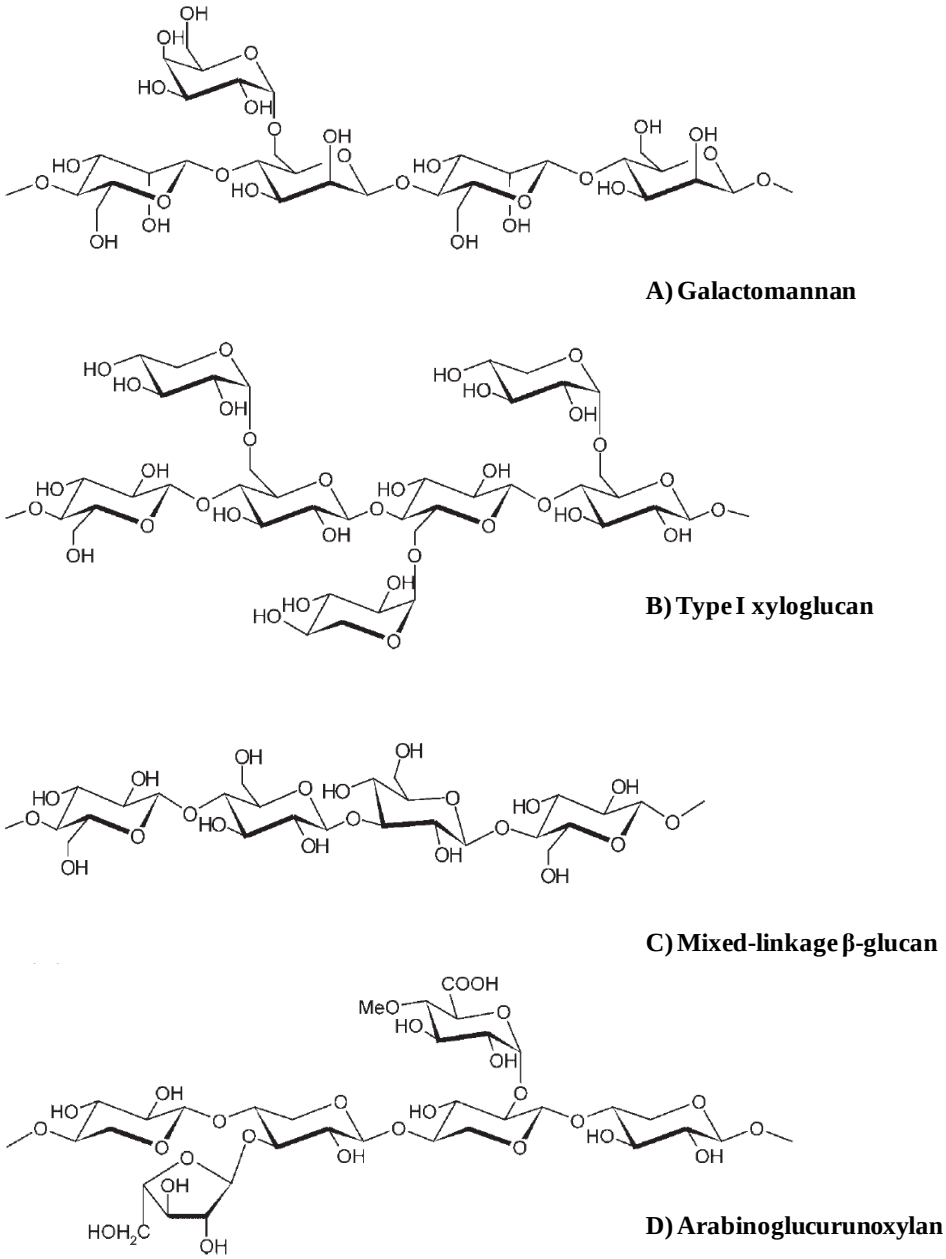


Fig. 1.7. Primary structures of common hemicelluloses. Adapted from Ebringerová (2006).

Mixed-linkage β-glucans (Fig. 1.7C) are a singular case because, unlike the rest of hemicelluloses, these polymers have an unbranched backbone. Chains are composed by blocks of β-1,4-glucopyranoses of variable length, separated by single β-1,3-glucopyranose residues. Cellulosic regions are mainly of cellotriose and cellotetraose, although uninterrupted sequences of up to 14 β-(1,4)-linked units can be found. These hemicelluloses are exclusively from *Flagellariaceae*, a family of monocotyledons which includes cereals, where mixed-linkage β-glucans are very abundant (Ebringerova, 2006; Scheller and Ulvskov, 2010).

Xylans are branched heteropolysaccharides formed by a backbone of β-(1,4)-linked xylopyranoses and different side chains. The abundance and

nature of the latter determine the denomination of the polysaccharide. Thus, glucuronoxylans display residues of α-D-glucuronic acid or 4-*O*-methyl-D-glucuronic acid linked to the *O*-2 positions of the main chain. When the polysaccharide also contains α-arabinofuranose it is called glucuronoarabinoxylan, arabinoglucuronoxylan or arabinoxylan if there is much more arabinose than uronic acid. In these cases, arabinose is linked to *O*-2 and/or *O*-3 positions of xylopyranose residues (Fig. 1.7D). Arabinoxylans usually display phenolic units, basically ferulic acid, attached to the *O*-5 position of arabinofuranose through an ester linkage. On the contrary, every type of xylan displays a certain grade of acetylation, which generally occurs at the *O*-3 position of xylose residues of the backbone and to a minor extent at *O*-2. When the global pattern of substitutions is more complex, including a series of mono- and oligosaccharides the polymer receives the name of heteroxylan. Xylans are the most abundant non-cellulosic plant polysaccharide, being the main hemicellulose in the secondary cell wall of dicotyledons and in cereal grains (Ebringerova, 2006; Scheller and Ulvskov, 2010).

1.4.3. Applications of biomass

Due to its low cost, abundance and structural and chemical diversity, the biotechnological potential of lignocellulosic biomass is considered to be huge, and it has been described as an inexhaustible source of energy and precursors (Cherubini and Stromman, 2011; Mabee et al., 2011). This raw material serves to obtain biofuels and analogs to petrochemicals, to induce enzyme production and even to culture edible microorganisms (Single Cell Protein) among other products of interest (Cherubini and Stromman, 2011; Kuhad et al., 2011).

In the last decade, most research has been focused on the production of second-generation bioethanol (2G), as an alternative to fossil fuels and first generation bioethanol (1G), which uses as raw material carbon sources suitable for human consumption. However, despite the fact that 2G bioethanol, which is produced from lignocellulosic biomass, avoids this issue, it displays its own drawbacks. The main limitation for production of 2G bioethanol is the need to remove or transform lignin in order to be able to use the plant cell wall polysaccharides (Martinez et al., 2009). At the same time, the exploitation of these carbohydrates requires more complex enzymatic cocktails than those used for 1G bioethanol, which is produced from easily hydrolysable sugars, as sucrose (in the case of sugar cane and beet) or starch (abundant in cereal grain) (Limayem and Ricke, 2012). Then, the high costs for producing 2G bioethanol make its current commercialization largely dependent on public policies of regulations and subsidies (Balat and Balat, 2009). The technological improvements of the productive process, together with the need of making 2G bioethanol

commercially viable have promoted the concept of biorefineries. The goal of this approach consists on the exploitation and valorization of lignocellulosic residues, using its potential beyond biofuels production (Bozell and Petersen, 2010; Martinez et al., 2009). Thus, in addition to bioethanol, plant wastes are used to obtain chemicals, proteins, biomaterials (Rødsrud et al., 2012), SCP (Mathews et al., 2011) or biohydrogen and biogas (Kaparaju et al., 2009) among others, making the global process profitable.

As previously stated, polysaccharides represent from 65 to 90% of lignocellulose (Betts et al., 1991), therefore glycosyl hydrolases are considered one of the most important enzymatic groups for its bioconversion (Hasunuma et al., 2013; Kumar et al., 2008; Sweeney and Xu, 2012).

1.5. XYLANOLYTIC ENZYMES: MAIN AND AUXILIARY ACTIVITIES

As discussed earlier, xylans constitute the second most abundant type of polysaccharide in the biosphere, which gives a first idea about their biotechnological relevance. Complete hydrolysis of these heteropolysaccharides requires the concerted action of multiple glycosidases and other CAZymes. The main enzymes involved in their degradation are endo-β-1,4-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37). Nevertheless, depending on the composition of the polysaccharide, these two GHs usually act together with α-L-arabinofuranosidases (EC 3.2.1.55), xylan α-1,2-glucuronosidases (EC 3.2.1.131), α-glucuronosidases (EC 3.2.1.139), acetylxylan esterases (AXEs) (3.1.1.72), feruloyl esterases (FAEs) (EC 3.1.1.73) (Shallom and Shoham, 2003) and the recently reported Rex enzymes (reducing-end-xylose releasing exo-oligoxylanase) (EC 3.2.1.156) (Honda and Kitaoka, 2004). These enzymes can be found in many organisms along the tree of life, as brown algae, protozoans, crustaceans, molluscs, insects, plants, fungi, eubacteria or archaebacteria (Michel et al., 2010; Polizeli et al., 2005). Each of these biocatalysts will be described in detail below.

1.5.1. Endo-β-1,4-xylanases

Endo-β-1,4-xylanases have been considered the most important enzymes in xylan hydrolysis since they were first described by Whistler and Masak (1955). Because of this, they are frequently denominated simply as xylanases. The main function of these biocatalysts is to hydrolyze the β-1,4 linkages between the residues of xylopyranose in the xylan backbone (Collins et al., 2005). Most of the known endoxylanases are encoded in

bacterial and fungal genomes, although they can be also found in plants, where they assume a role in the remodeling of the cell wall (Suzuki et al., 2005). Fungal and bacterial xylanases are produced, in variable amounts, in the presence of any lignocellulosic residue (Beg et al., 2001), but their maximum expression is usually achieved upon induction by xylan or substrates enriched in this polysaccharide in the culture medium.

The endoxylanase activity is widely distributed among the CAZy families, with representatives in GH5, GH8, GH10, GH11, GH30, GH43, GH51 and GH98, therefore its diversity at the structural, physico-chemical and specificity level is not unexpected. Among them, only the enzymes from families 8, 43 and 98 are inverting glycosidases whereas the others operate by double displacement (www.cazy.org/). The most studied endoxylanases belong to families 10 and 11 (Collins et al., 2005; Polizeli et al., 2005).

Structurally, the endoxylanases from family GH10 contain from four to five subsites (Berrin and Juge, 2008) and display a $(\beta/\alpha)_8$ barrel fold for the active module. This conformation is usually compared to a "salad bowl", with the active site located at the narrower end, near the C-terminal of the β-barrel (Collins et al., 2005; Jeffries, 1996). Regarding to their physico-chemical properties, these endoxylanases show higher molecular mass (>30 kDa) and lower isoelectric point (pI) than the enzymes from family 11 (Collins et al., 2005; Päes et al., 2012). GH10 enzymes are more versatile and efficient than their GH11 counterparts when applied to the hydrolysis of heteroxylans. This fact has been related to their capacity for hydrolyzing β-1,4 linkages between xylopyranose residues located in the surroundings of some of the frequent xylan substituents, as methyl-glucuronic or acetic acids. In the same way, the (uncommon) presence of β-1,3 linkages in the main chain affect much more negatively GH11 endoxylanases than GH10 (Biely et al., 1997). Indeed, there are xylanases from family 10 with the ability of hydrolyzing these β-1,3 glycosidic bonds between xylopyranoses (Chen et al., 1986) and even examples displaying activity on cellulosic substrates (Kim et al., 2009).

Endoxylanases belonging to family 11 adopt a β-jelly roll fold and most of them contain five subsites. These biocatalysts show lower molecular mass (<30 kDa) and basic pI, and usually share a wide range of pH tolerance (Päes et al., 2012). Their selectivity is much more strict than the previous case, hydrolyzing exclusively β-1,4 linkages and being strongly inhibited by the proximity of substituents or β-1,3 glycosidic bonds. These glycosidases are only able to act on regions of at least three unsubstituted β-(1,4)-linked xylopyranose residues (Biely et al., 1997). This family had been considered an example of monospecificity (something infrequent) for grouping endo-β-1,4-xylanases exclusively, although a bifunctional enzyme displaying endo-1,3-β-xylanase (EC

3.2.1.32) activity has been recently added (www.cazy.org). Due to their high specificity, these GHs are sometimes called "true xylanases" and they typically hydrolyze xylan in larger products than GH10 endoxylanases do, which, in fact, can use the xylooligosaccharides released by GH11 glycosidases as their own substrates (Collins et al., 2005).

Endoxylanases from other families are, at the moment, less studied, but some of them display promising properties. GH5 enzymes hydrolyze efficiently different polysaccharides apart from xylan, as lichenan or carboxymethylcellulose. There are few examples of GH8 endoxylanases, but the enzyme produced by *Pseudoalteromonas haloplanktis*, TAH3a, is remarkable due to its capacity for specifically hydrolyzing xylan at low temperatures in an efficient way (Collins et al., 2005). At the structural level, it has been reported that the catalytic module of GH8 glycosidases adopts an alpha helix $(\alpha/\alpha)_6$ barrel fold. Family GH30 groups endoxylanases that are considered to be specialized in the hydrolysis of glucuronoxylans, usually requiring the presence of a methyl-glucuronic substituent in the surroundings of the target linkage in the main chain (Urbániková et al., 2011). Families 43 and 51 contain few examples of characterized endoxylanases and all reports describe bifunctional glycosidases with α-L-arabinofuranosidase activity (Collins et al., 2005; Giacobbe et al., 2014; Yang et al., 2015). GH30 and GH51 glycosyl hydrolases share a $(\beta/\alpha)_8$ barrel fold in the module containing the active site, whereas GH43 enzymes display a 5-fold β-propeller structure. Family 98 is understudied and it contains only one characterized endoxylanase in CAZy, without an assigned 3D folding. However, this enzyme can be highlighted because its producer, *Bacteroides ovatus*, is a commensal organism in the human gut microbiota (Rogowski et al., 2015; Whitehead, 1995).

1.5.2. Exo-1,4-β-D-xylosidases

Exo-1,4-β-D-xylosidases, generally known as β-xylosidases, play, together with endoxylanases, the central role in the hydrolysis of xylan. These enzymes use as substrates the hydrolytic products released by endoxylanases and convert them into D-xylose. β-xylosidases hydrolyze the β-1,4 linkage between xylopyranose units of xylobiose (xylose disaccharide) and other XOS. They are exo-glycosidases and act from the non-reducing end of oligosaccharides, releasing a unit of monosaccharide each time. As in the case of endoxylanases, β-xylosidases are produced by bacteria, fungi and plants, although the enzymes applied at the industrial level are mainly from the first two groups (Jordan and Wagschal, 2010; Kousar et al., 2013). The exo-1,4-β-D-xylosidase activity is even more widespread in CAZy than endo-β-1,4-xylanase. Thus, GH1, GH3, GH30, GH39, GH51, GH52, GH54, GH116 and GH120 families include the

retaining β-xylosidases, while the inverting enzymes belong to family 43 (www.cazy.org). Most bacterial β-xylosidases are found in families 39 and 43 (Kousar et al., 2013), whereas their fungal counterparts have been exclusively classified as GH3, GH43 and GH54 (Knob et al., 2010). Unlike endoxylanases, which are extracellular proteins, there are examples of intracellular β-xylosidases, located both in cytosol and periplasm (De Almeida et al., 1995; Katapodis et al., 2006; Ohta et al., 2010), and although most of them have been found in yeast and bacteria, there are also intracellular glycosidases with this activity produced by filamentous fungi (Knob et al., 2010). Until now GH3 and GH43 β-xylosidases have been the most studied and commercially applied.

GH3 exo-1,4-β-D-xylosidases can be found in archaebacteria, eubacteria or plants, but the family stands out for containing a great number of fungal β-xylosidases (Kousar et al., 2013). In spite of being one of the largest (12,854 deposited sequences) and most studied families in CAZy, there is, at the moment, very little information available at the structural level, and no general folding has been proposed for the catalytic module of this family (Lagaert et al., 2014). The examples of bifunctional enzymes are frequent, especially those which associate β-glucosidase and β-xylosidase activities (Faure, 2002). In general terms, these are proteins encoded by genes of more than 2,000 bp (Knob et al., 2010), which are translated constituting monomers around 80-100 kDa, commonly associated in dimeric active forms (Dilokpimol et al., 2011; Eneyskaya et al., 2007). Optimum pH is usually from 4 to 6 and the enzymes display maximal activity at 50-60 ºC (De Almeida et al., 1995; Wongwisansri et al., 2013), although some thermophilic GH3 β-xylosidases haven been also reported (Shi et al., 2013). At the catalytic level, glycosidases from this family are outstanding for their high substrate affinity (Eneyskaya et al., 2007; Zhou et al., 2012) and, particularly, fungal proteins frequently show α-L-arabinofuranosidase side activity (Mozolowski and Connerton, 2009). Besides their hydrolytic properties, these biocatalysts are known to catalyze transglycosylation reactions, which in this case receive the name of transxylosylation. GH3 β-xylosidases with this capacity usually display certain versatility, being able to use a wide variety of acceptors and successfully catalyze the transfer of a xylopyranose residue not only to xylooligosaccharides and xylose itself, but also to different monosaccharides, disaccharides, aliphatic alcohols, sugar alcohols or phenolic derivatives (Dilokpimol et al., 2011).

β-xylosidases from family 43, due to their condition of inverting glycosidases, are considered the most suitable for the efficient saccharification of lignocellulosic materials, as the inability to catalyze transglycosylation keeps their hydrolytic activity even in the presence of high concentration of substrate (Jordan et al., 2007). Enzymes from this

family adopt a 5-fold β-propeller fold and show a third acid residue, highly conserved and necessary for catalysis, which acts modulating the pKa of the catalytic acid/base, helping this residue to get the right orientation respect to the substrate (Brüx et al., 2006). GH43 β-xylosidases have been found in archaebacteria, eubacteria, plant and fungi, although bacterial enzymes are the most common (Kousar et al., 2013; www.cazy.org). The industrial relevance of this family led to an in-depth characterization of many members, revealing a wide diversity and allowing Mewis *et al*. (2016) to establish 37 subfamilies: ten of them contain β-xylosidases, frequently together with α-L-arabinofuranosidases and, in many occasions, enzymes displaying both activities are found. Some GH43 β-xylosidases show a xylan binding domain denominated CBM6 in CAZy classification, a typical module in glycosidases acting on insoluble substrates, which may explain the frequent reports on β-xylosidases directly active against xylan in this family (Kousar et al., 2013; Mewis et al., 2016).

Family 39 comprises a relatively small group of sequences and, until now, it does not include any eukaryotic β-xylosidase. Among the bacterial enzymes, several proteins capable of catalyzing transxylosylation have been noticed, although displaying low regioselectivity. Its main structural characteristic is the $(\beta/\alpha)_8$ barrel fold of the active module, and some of these enzymes show a tetrameric quaternary structure (Lagaert et al., 2014).

Characterized GH51 and GH54 glycosidases are mainly α-L-arabinofuranosidases, although a single example of a bifunctional enzyme displaying also β-xylosidase activity has been reported in both families. On the contrary, regarding to family 52, the nine characterized enzymes which are currently known are β-xylosidases without showing any other activity (Lagaert et al., 2014). The available information about members of families 116 and 120 is even more limited. A total of five characterized enzymes are included in GH116, and only one of them, produced by the thermophilic archaebacterium *Sulfolobus solfataricus*, has demonstrated β-xylosidase activity (Cobucci-Ponzano et al., 2010). GH120 contains two enzymes classified as β-xylosidases and one of them is encoded at the genome of the human probiotic *Bifidobacterium adolescentis* (Lagaert et al., 2011). At the structural level, the members of GH51 adopt a $(\beta/\alpha)_8$ barrel fold, whereas, at this moment, there is no 3D structure associated to families 52, 54, 116 and 120 in CAZy.

1.5.3. Auxiliary activities

As mentioned at the beginning of this section, xylan is a heteropolysaccharide whose complexity makes insufficient the activity of endoxylanases and β-xylosidases for achieving its complete hydrolysis. The efficient degradation of this polymer requires the participation of a

series of enzymes displaying activities that can be considered as auxiliary (do not confuse with AAs class in CAZy) and will be described below.

α-L-Arabinofuranosidases are glycosidases that hydrolyze the linkage between an α-L-arabinofuranosyl substituent and a xylose unit of the xylan backbone. These enzymes are of the exo-type and act from the non-reducing end of their substrate releasing arabinofuranose units, being able to hydrolyze α-1,2, α-1,3 and α-1,5 linkages. α-L-Arabinofuranosidases are distributed in CAZy among families GH2, GH3, GH43, GH51, GH54 and GH62, therefore many of characterized α-L-arabinofuranosidases share group with endoxylanases and β-xylosidases (Juturu and Wu, 2013; Saha, 2000). Bacteria and filamentous fungi are the sources of the majority of the characterized enzymes displaying this activity and their expression in cultures is induced by biomass derivatives. Although arabinose and arabitol are usually the best inducers, high levels of this activity have also been reported in the presence of xylans, xylose or xylitol. In nature, there are intra- and extracellular α-L-arabinofuranosidases and their expression is frequently regulated in concert with arabinases (EC 3.2.1.99) which are endo-type enzymes acting on another class of hemicelluloses, the arabinans (Saha, 2000). From the perspective of CAZy families, GH51 stands out for containing the largest number of α-L-arabinofuranosidases, almost 70 proteins from bacteria and fungi. Families 43 and 54 also include a high number of characterized enzymes, but the former contains exclusively bacterial members displaying this activity whereas the latter groups only fungal α-L-arabinofuranosidases (www.cazy.org).

Xylan α-1,2-glucuronidases (EC 3.2.1.131) and α-glucuronidases (EC 3.2.1.139) hydrolyze the glycosidic bond between glucuronic acid or its 4-*O*-methylglucuronic derivative and the xylopyranose units of the xylan main chain. The fact that this activity is defined by two different EC numbers might be confusing, as sometimes the same enzyme is associated to both identifiers. This is the case of α-glucuronidase from *Aspergillus niger* (encoded by *agua* gene), which can be found in the literature as an EC 3.2.1.139 enzyme (Stricker et al., 2008), but it is deposited in CAZy under the EC 3.2.1.131 number. According to the ENZYME database (http://enzyme.expasy.org/) the difference between these classifications is based on the higher substrate diversity found for EC 3.2.1.139, whereas EC 3.2.1.131 glycosidases hydrolyze exclusively α-1,2 linkages in xylan. Both GHs will be called from now on α-glucuronidases, which is the most frequent term in literature. This activity is found in families GH4, GH67 and GH115, although GH4 is no relevant in the context of this thesis, as the only α-glucuronidase characterized within this group is inactive against xylan and its degradation products (Suresh et al., 2003; Ryabova et al., 2009). Remarkable differences have been reported among GH67 and

GH115 α-glucuronidases. As family 67 has been more thoroughly studied it also contains more characterized enzymes, as it is indicated in CAZy. Glycosidases belonging to this family are not active against the polymer of xylan, but they act on the glucuronoxylan fragments released and their target is a glucuronic acid substituent attached to the xylopyranose residue of the non-reducing end. These types of oligosaccharides are released by the activity of GH10 endoxylanases and receive the name of alduronic acids. This limitation, together with the high number of intracellular enzymes reported, has led to propose, at least for prokaryotic α-glucuronidases, that their role is to act on xylan branches once the hydrolytic products are transported inside the cell, where β-xylosidases can complete the full degradation to xylose. GH115 α-glucuronidases belong to a small family recently created, and unlike the GH67 enzymes, they can act directly on the complete polysaccharide, hydrolyzing 4-*O*-metilglucuronic substituents at any position of the chain (Juturu and Wu, 2013; Ryabova et al., 2009).

Acetylxylan esterases are xylanolytic enzymes which do not belong to the class of glycosyl hydrolases, but to carbohydrate esterases group. AXEs catalyze the hydrolysis of ester linkages between a xylopyranose residue and its acetyl substituent. These enzymes have also a wide distribution in the CAZy classification, being found in families CE1, 2, 4, 5, 6, 7 and 12, which indicates a notable sequences' divergence (Juturu and Wu, 2013; Topakas and Paul, 2007). The main sources of acetylxylan esterase activity are filamentous fungi and bacteria and the maximal production is induced by the presence of xylans or complex lignocellulosic materials (Christov and Prior, 1993). Currently, most of the characterized fungal and bacterial AXEs belong to families 1 and 4, respectively (Topakas and Paul, 2007). Regardless of their origin, several studies have demonstrated a synergic effect on xylan hydrolysis when endoxylanases and β-xylosidases act together with acetylxylan esterases (Kosugi et al., 2002; Suh and Choi, 1996). Interestingly, in spite of being enzymes involved in xylan degradation, its expression seems to be controlled both by the cellulolytic and hemicellulolytic systems (Christov and Prior, 1993).

Feruloyl esterases are a different group of CEs whose function is to hydrolyze ferulic acid residues esterified to some arabinoses in the side chains of xylan. These enzymes belong exclusively to family CE1 (Juturu and Wu, 2013) and, according to CAZy, their catalytic module adopts a (α/β/α) sandwich fold. Many lignocellulolytic organisms, both fungi and bacteria, express these enzymes, and maximal levels are induced by complex carbon sources (lignocelluloses), being uncommon their production in the presence of sugar alcohols, monosaccharides or disaccharides (Topakas et al., 2007). Although the classical definition of this enzymatic group only refers to ferulic acid, currently, the name

feruloyl esterases groups enzymes with the ability of releasing other types of phenolic acids. Attending to their targets and protein sequences, Crepin *et al*. (2004) established four FAEs subtypes. Enzymes classified into the A subtype act preferentially on phenolic derivatives displaying methoxy substituents, especially at para- position, as ferulic and sinapic acids, and can only hydrolyze esters at the *O*-5 position of L-arabinofuranose. FAEs from the B subtype show higher affinity against phenolic rings substituted by one or two hydroxyls, being *p*-coumaric and caffeic acids the most frequent examples, and hydrolyze ester linkages both at *O*-5 and *O*-2 positions. Enzymes from subtypes C and D share a wide specificity against methyl derivatives of the phenolic acids mentioned above. These groups differ in their capacity of hydrolyzing diferulates, which is absent in the B and C subtypes, but present in A and D. It must be highlighted that, in recent years, this classification has been deepened and now it includes a total of twelve subtypes, but the initial division in four is still frequently used (Gopalan et al., 2015). Activity on diferulates gives special relevance to these enzymes, because dimmers of ferulic acid may form bonds between lignin and the polysaccharide components of the plant cell wall (Buanafina, 2009; Topakas et al., 2007).

The Rex activity, also known as exo-oligoxylanase, was first reported in 1994 (Kubata et al., 1994), although a Rex enzyme was not isolated and characterized until 2004 (Honda and Kitaoka, 2004). Rex enzymes act releasing xylopyranose units from the reducing-end of a xylooligosaccharide containing at least three residues. They are inactive against xylans and their activity release, in addition to xylose, xylobiose as final product (Juturu and Wu, 2014). According to CAZy, exo-oligoxylanases belong exclusively to the GH8 family and, structurally, their catalytic module adopts an $(\alpha/\alpha)_6$ barrel fold. Up to date all characterized Rex enzymes are from bacteria and, although the number of available sequences is very low, these glycosidases are produced in a considerable variety of environments. Thus, they have been found in bacteria inhabiting cattle rumen, soil, thermal waters and even in the Antarctic station, and it is remarkable that some of the producer microorganisms are probiotic species from the *Bifidobacterium* genus (Juturu and Wu, 2014). Recently, it has been settled that these glycosidases also act on branched xylooligosaccharides (Valenzuela et al., 2016), which would reinforce the concerted action of endoxylanases and exo-oligoxylanases. This synergism is the reason of the great biotechnological potential of Rex enzymes.

Table 1.2 summarizes the characteristics of the main enzymes described in sections 5.1, 5.2 and 5.3. The 3D structure of the catalytic module associated to each family is included, together with a reported structure as an example. 3D structures were obtained from RCSB Protein Data Bank and elaborated using the Pymol software (v1.8.4.0).

Table 1.2. Main xylanolytic enzymes and summary of their properties.

Enzyme	EC number	Family	Folding	Example		
				Structure	Organism	PDB ID
Endoxylanase	3.2.1.8	GH10	$(\beta/\alpha)_8$ barrel		*Penicillium simplicissimum*	1B30
		GH11	β-jelly roll		*Trichoderma reesei*	1ENX
β-Xylosidase	3.2.1.37	GH3	NIA		*Trichoderma reesei*	5A7M
		GH43	5-fold β-propeller		*Bacillus halodurans*	1YRZ
α-L-Arabinofuranosidase	3.2.1.55	GH43	5-fold β-propeller		*Halothermothrix orenii*	4QQS
		GH51	$(\beta/\alpha)_8$ barrel		*Bifidobacterium longum*	2Y2W

		GH54	NIA		*Aspergillus kawachii*	1WD3
α-Glucuronidase	3.2.1.131 3.2.1.139	GH67	$(\beta/\alpha)_8$ barrel		*Cellvibrio japonicus*	1GQI
		GH115	NIA		*Bacteroides ovatus*	4C90
Acetylxylan esterase	3.1.1.72	CE1	$(\alpha/\beta/\alpha)$ sandwich		NIA	
		CE4	$(\beta/\alpha)_7$ barrel		*Streptomyces lividans*	2CC0
Feruloyl esterase	3.1.1.73	CE1	$(\alpha/\beta/\alpha)$ sandwich		*Ruminiclostridium thermocellum*	1JJF
Exo-oligoxylanase	3.2.1.156	GH8	$(\alpha/\alpha)_6$ barrell		*Bacillus halodurans*	1WU4

NIA: No information available

1.5.4. Xylan hydrolysis: a concerted action

Once established the main activities participating in xylan degradation it is possible to formulate a general model to describe the full hydrolytic process. However, it should be emphasized that not every lignocellulolytic organism express all the described enzymes and these are not produced at the same level, being some of them low or residual. Nevertheless, in nature, the degradation of this polysaccharide is not a process carried out by an isolated organism, but also the result of the concerted action of biological consortia, where different fungal and bacterial species can be found (Zuroff and Curtis, 2012). It is also necessary to remember that xylan, together with cellulose and lignin, is one of the basic components of plant biomass, which may explain the strong interrelation between cellulolytic, xylanolytic and ligninolytic metabolisms (Ander, 1994; Brown et al., 2014).

Currently, the expression of xylanolytic enzymes is considered to be regulated through the xylan degradation products themselves. The most accepted model involves the existence of certain constitutive endoxylanases which, despite of being produced at low level, are necessary for starting hydrolysis. Once these enzymes come into contact with xylan, the XOS released are transported to the intracellular space inducing the expression of the whole xylanolytic system, including the endoxylanase activity itself (Collins et al., 2005). This induction can be independent of the cellulolytic metabolism, but in some organisms it is interrelated. In this sense, it has been reported that glucose-derived compounds can activate the expression of cellulases and xylanolytic enzymes (Haltrich et al., 1996).

Once the hemicellulolytic system is induced, but the process is still in the first stages of degradation, some auxiliary enzymes act on xylan besides endoxylanases removing substituents from the xylan backbone, as it is the case of GH115 α-glucuronidases. As the polysaccharide is highly decorated, GH11 endoxylanases find few available targets, so they act releasing products of high molecular mass, which are then substrates for GH10 xylanases. The progression of the degradation reduces the branching degree of the xylan, facilitating the action of GH11 endoxylanases and the production of shorter xylooligosaccharides that can be further degraded extracellularly or after entering the cytosol. At this point, Rex enzymes can participate in the process, hydrolyzing XOS into xylose and xylobiose, and β-xylosidases complete the hydrolysis by releasing exclusively the monosaccharide. The auxiliary enzymes act simultaneously to the former glycosidases, removing the remaining substituents of the oligosaccharides released by xylanases.

Figure 1.8 schematizes and summarizes the current proposed model of xylan hydrolysis.

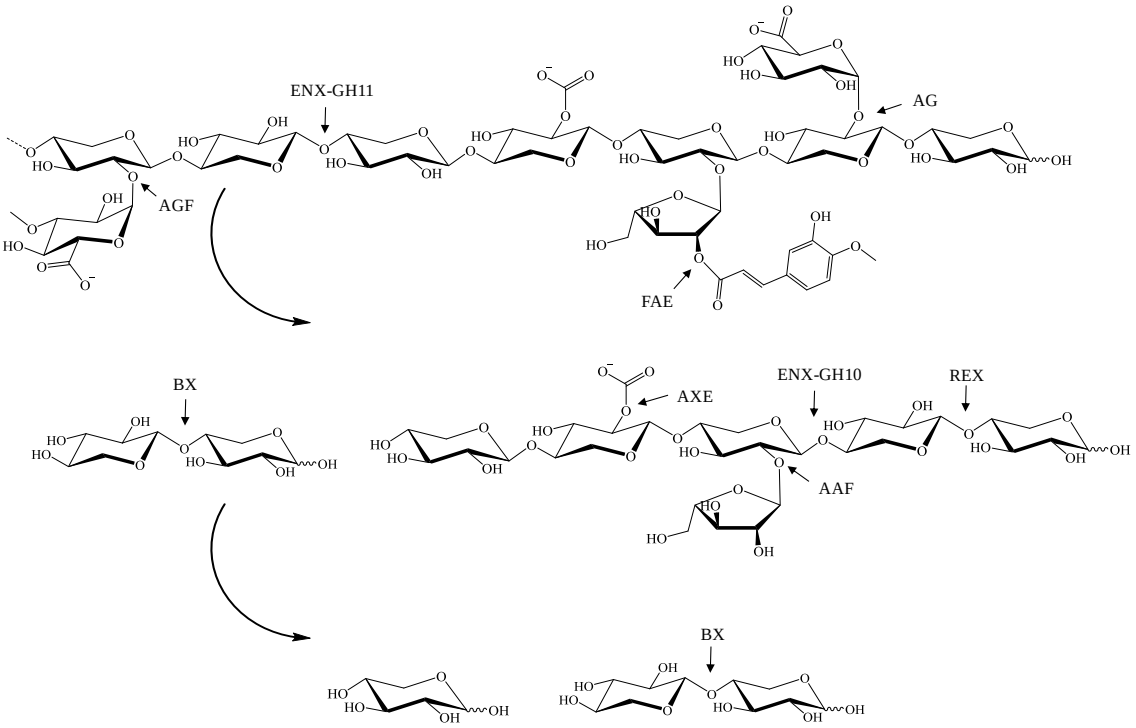


Fig. 1.8. Basic model of enzymatic hydrolysis of xylan. AAF: α-L-arabinofuranosidase; AG: α-glucuronidase; AXE: acetylxylan esterase; BX: β-xylosidase; FAE: feruloyl esterase; ENX: endoxylanase; REX: exo-oligoxylanase.

1.6. FILAMENTOUS FUNGI AS SOURCES OF XYLANOLYTIC ENZYMES

Bacteria and fungi are the main producers of xylanolytic enzymes in nature. The use of prokaryote organisms entails certain advantages over the eukaryotic sources (fungi in this case) both at the research and industrial levels. In this sense, genetic manipulation of bacteria and scale-up of their cultures are commonly simpler. In addition, bacterial enzymes generally lack post-translational modifications, displaying less difficulties than eukaryotic proteins when produced in prokaryotic expression systems, which are the most commonly used (Terpe, 2006). However, in spite of these and other advantages, the commercial interest has been focused mainly on fungal xylanases, which have been more extensively studied. One of the essential reasons for this is the fact that filamentous fungi produce much higher levels of xylanolytic activities than those reported for bacteria and yeasts. Additionally, most bacterial glycosidases are intracellular or periplasmic, whereas fungal xylanases usually enter in the secretion pathway and are released to the extracellular medium (Polizeli et al., 2005).

Among filamentous fungi, the phylum Ascomycota is considered as the main producer of enzymes involved in the degradation of cellulose and hemicellulose. In particular, *Trichoderma* and *Aspergillus* have been

thoroughly studied for this application and their enzymes are used at the industrial level, although several species from the genus *Penicillium* have emerged in the last decade as promising sources of hemicellulases with great biotechnological potential (Chavez et al., 2006). The three genera group imperfect (anamorphic) states, in other words, fungi without known sexual reproduction. When the mechanism of sexual reproduction is reported for a species from any of these genera its nomenclature is modified and it is transferred to the appropriate associated teleomorphic (sexual) genus. Taking into account that taxonomy changes along time, it is usual to find in literature different denominations which are considered synonyms for a unique microorganism. Some examples of this will be noticed below, with the description of the hemicellulolytic potential of each genus.

1.6.1. The genus *Trichoderma*

Currently, fungi from the genus *Trichoderma* are classified as *Hypocrea* spp. once their sexual state is reported. The most remarkable species of the genus is *Trichoderma reesei*, a saprophyte which constitutes the main source of cellulases and xylanolytic enzymes at the industrial level and has been renamed as *Hypocrea jecorina* some years ago (Mach and Zeilinger, 2003; Martinez et al., 2008). The xylanolytic machinery of this fungus consists of different types of activities and it is gathered in Table 1.3.

It is worth mentioning that in spite of the apparent complexity of this system, 90% of the xylanolytic activity is due exclusively to the action of two enzymes, the GH11 endoxylanases XYN1 and XYN2. The fact that both glycosidases are active in a limited range of conditions, together with their low stability, are limiting factors for the potential of this fungus as a biotechnological tool for xylan hydrolysis (Törrönen et al., 1992).

In addition, it has been reported that the use of *T. reesei* enzymatic cocktails for the saccharification of lignocellulose causes the accumulation of xylooligosaccharides. XOS have revealed to be strong inhibitors of cellulases and consequently produce a decrease on the global yields of the process (Qing et al., 2010). This organism also lacks the necessary enzymes for the efficient hydrolysis of pectins or other types of hemicelluloses as arabinans (Bischof et al., 2016). In order to compensate these deficits, cocktails of *T. reesei* have been supplemented with other preparations enriched in hemicellulases for years (Berlin et al., 2006). However, there is still room for improvement, so the search for novel fungi producing xylanolytic enzymes remains as a topic of interest nowadays. These efforts have led to the characterization of hemicellulases from other *Trichoderma* species (Toth et al., 2013), among which *Trichoderma*

longibrachiatum stands out (Azin et al., 2007; Royer and Nakas, 1989), and the search has been extended to other filamentous fungi, yeast and bacteria.

Table 1.3. Xylanolytic system of *Hypocrea jecorina*: activities, CAZy families and number of encoded enzymes in each case.

Activity	Family	Encoded enzymes	Reference
Endoxylanase	GH10	1	(Häkkinen et al., 2012)
	GH11	3	
β-xylosidase	GH30	2	
	GH3	1	
	GH39	1	
β-xylosidase/α-L-arabinofuranosidase	GH43	2	
α-L-arabinofuranosidase	GH54	2	
	GH62	1	
α-glucuronidase	GH67	1	
	GH115	1	
AXE	CE3	2	
	CE5	3	
4-*O*-methyl-glucuronyl methylesterase	CE15	1	(Li et al., 2007)

1.6.2. The genus *Aspergillus*

Aspergillus is one of the major genera of ascomycetes for the high number of known species that it groups, comprising from human pathogens to many saprophytic species that are interesting for secreting high levels of xylanolytic enzymes. *Aspergillus aculeatus*, *Aspergillus awamori*, *A. niger* or *Aspergillus oryzae* are some examples of species studied as producers of endoxylanases, mainly from families 10 and 11, and β-xylosidases, generally from GH3 (De Vries and Visser, 2001), being *A. niger* KK2 a remarkable producer of the latter activity (Kang et al., 2004). It must be noticed that *A. niger* constitutes a source of thermostable endoxylanases and β-xylosidases (Coral et al., 2002; Pedersen et al., 2007), but the relevance of this species is not only due to its capacity of producing lignocellulolytic enzymes (Kang et al., 2004). This organism is also widely used as heterologous host for the expression of proteins of interest (Ward, 2012) for its high levels of secretion of extracellular enzymes and for possessing the GRAS status (Generally Regarded As Safe) (Schuster et al., 2002), which makes it one of the most studied members of this genus.

As just mentioned, β-xylosidases are key enzymes for the successful saccharification of xylan, and they are produced in high levels by fungi

from the genus *Aspergillus*. Furthermore, these glycosidases have a special relevance because they catalyze transxylosylation reactions. Although this ability has been also noticed for enzymes from *Trichoderma* species, the number of reports on this activity is very limited and restricted to the synthesis of alkylxylosides (Drouet et al., 1994; Pan et al., 2001). On the contrary, the more versatile β-xylosidases from some aspergilli have been applied to the synthesis of xylosides derived not only from simple alkanols, but also from phenols, sugar alcohols, monosaccharides or disaccharides (Kurakake et al., 1997; Sulistyo et al., 1995).

Apart from endoxylanases and β-xylosidases, *A. niger* (together with many other members of this genus) also expresses auxiliary xylanolytic enzymes, and notably, besides α-L-arabinofuranosidase, α-glucuronidase and acetylxylan esterase, it produces feruloyl and coumaroyl esterase activities, lacking in *T. reesei* (De Vries and Visser, 2001).

Some species, as *Aspergillus terreus* and *A. niger*, produce high levels of endoxylanase with negligible quantities of cellulases in solid state fermentation using lignocellulosic materials as carbon source, which is a remarkably low-cost approach. Although this could be a drawback for saccharification of plant biomass, where cellulases are essential, this cocktail without cellulolytic activities is especially suitable for the bleaching of paper pulp (Gawande and Kamat, 1999). In the same way, certain *Aspergillus* species can degrade other polysaccharides of the plant cell wall, as pectins, making them very suitable for biomass deconstruction. This is the case of *A. niger*, from which at least ten pectinases have been characterized (De Vries and Visser, 2001).

1.6.3. The genus *Penicillium*

Penicillium groups more than 250 different members widely distributed in nature (Gusakov and Sinitsyn, 2012). The taxonomic status of its species and those belonging to any of its associated sexual states is not fully established. Due to its relevance in this doctoral thesis, only *Talaromyces* will be mentioned as one of the genera where some perfect states have been traditionally included (Samson et al., 2011).

Many *Penicillium* strains, especially those living as soil saprophytes, are strong producers of xylanolytic activities. Upon a bibliographic survey, Chavez *et al*. (2006) found reports of 27 species of the genus applied to the production of endoxylanases and/or β-xylosidases. A year later, an assay performed on over one hundred *Penicillium* isolates was reported, finding 85 which produced endoxylanase activity, 33 of them with remarkably high levels (Yoon et al., 2007). A similar analysis, but focused on 36 *Penicillium* species isolated from wood, settled that many of these fungi were producers of both endoxylanases and β-xylosidases and, although the latter activity was not detected in all of them, novel sources of both

enzymes were found (Lee et al., 2012). The search of members of the genus with unknown xylanolytic abilities continues nowadays and, as an example of this, a very recent report describes *Penicillium subrubescens* as an organism able to saccharify biomass to a similar extent as *A. niger* (Mäkela et al., 2016).

Besides their potential as producers, *Penicillium* species secrete xylanolytic enzymes showing interesting properties. In this sense, the study of Berlin *et al*. (2006) suggests that these enzymes might display an inferior rate of non-productive bindings to lignin, in comparison to the enzymatic complexes from *Trichoderma* or *Aspergillus*, which would lead to higher saccharification yields for lignocellulosic substrates. Moreover, species as *Talaromyces emersonii* and *Talaromyces thermophilus* produce thermostable endoxylanases, β-xylosidases and xylanolytic auxiliary enzymes (Ben Romdhane et al., 2010; Guerfali et al., 2009; Tuohy et al., 1994), whereas *Penicillium citrinum* and *Penicillium* sp. SS1 are sources of active and stable endoxylanases at alkaline pH, which is a desired property for the paper industry (Bajaj et al., 2011; Dutta et al., 2007). Another endoxylanase from *Penicillium pinophilum*, XYN10C1, combines thermostability and tolerance to acid pH (Cai et al., 2011), and *Talaromyces stipitatus* constitutes a very interesting case for producing a set of three different feruloyl esterases, one of them considered unique because of its wide substrate specificity (Vafiadi et al., 2006).

On the contrary, transxylosylation is a less studied activity in this genus, in comparison to *Aspergillus*, although there are reports on β-xylosidases from *Penicillium* with the ability to catalyze the synthesis of alkylxylosides (Ito et al., 2003) and xylooligosaccharides (Win et al., 1988). The β-xylosidase from *T. thermophilus* outstands for its ability to synthesize xylooligosaccharides of up to four units from xylose by reverse hydrolysis, an activity more infrequent than transxylosylation itself (Guerfali et al., 2009).

Beyond the particular issue of xylanolytic activities and upon an overall comparison, several *Penicillium* species produce similar levels of saccharification or even higher than *T. reesei* depending on the chosen lignocellulosic substrate (Chekushina et al., 2013; Marjamaa et al., 2013). One of the most interesting species from a biotechnological perspective is *Penicillium oxalicum*, previously known as *Penicillium decumbens* (Liu et al., 2013). It is a soil saprophyte, isolated for the first time in 1915 (Currie and Thom, 1915), used by the Chinese industry for large-scale production of cellulases. Besides its high production of cellulolytic enzymes, *P. oxalicum* surpasses *T. reesei* for releasing enzymatic preparations with a better balance of (hemi-)cellulolytic activities and high xylanolytic activity. Genomic analyses have concluded that this organism encodes a remarkably higher number of CAZymes than *T. reesei* and other penicillia as

Penicillium chrysogenum for hydrolyzing cellulose, xylans and pectins. At the same time, it contains a lower number of genes involved in cellular metabolism and regulation. This superior hydrolytic potential is in good agreement with secretome analyses that disclosed greater levels and diversity of hemicellulolytic activities in *P. oxalicum* (Liu et al., 2013), encoding six different endoxylanases from families GH11, GH10 and GH30. These endoxylanases can be easily distinguished at the biochemical level on the basis of their temperature and pH profiles, substrate specificity and catalytic parameters. In addition, the six glycosidases are differently induced depending on the selected carbon source. This diversity highlights the potential of *P. oxalicum* as a biotool for the degradation of complex hemicelluloses (Liao et al., 2015).

However, other isolates from *Penicillium* or its teleomorphs have demonstrated their biotechnological potential in recent years, as it is the case of *T. stipitatus* (Vafiadi et al., 2006), *Talaromyces cellulolyticus* (Fujii et al., 2014), *Talaromyces verruculosus* (Goyari et al., 2015) or *Talaromyces amestolkiae* (Gil-Muñoz, 2015), the microorganism used in the present work as enzyme producer, that is briefly introduced below.

1.6.4. *T. amestolkiae*: a novel and promising source of glycosidases

T. amestolkiae strain CIB was isolated from cereal wastes and identified in the group of Professor María Jesús Martínez (Centro de Investigaciones Biológicas, CIB-CSIC). This organism was characterized for its cellulolytic potential, displaying remarkable levels of β-glucosidase activity. Most commercial lignocellulolytic cocktails, and especially those obtained from *T. reesei*, are deficient in this enzymatic type (Gritzali and Brown, 1979). This feature made the selection and study of this microorganism particularly interesting, as *T. amestolkiae* crudes can be good candidates to supplement commercial preparations extensively used as Celluclast 1.5L or Ultraflo L. In fact, the results of saccharification experiments using with either this crude or the commercial NS50010 cocktail as supplements of β-glucosidase activity were similar or even better with *T. amestolkiae*'s crudes. Currently, three β-glucosidases secreted by this fungus have been purified and characterized, one belonging to family GH1 and two to family GH3. These enzymes stand out because of their high affinities against *p*-nitrophenyl β-D-glucopyranoside and for their catalytic efficiency against natural substrates as cellobiose, superior to those reported for other β-glucosidases. In addition to their hydrolytic properties, the three enzymes can catalyze transglycosylation reactions and were successfully tested in the synthesis of cellooligosaccharides and alkylglycosides. Regarding to this latter group of compounds, it should be emphasized that the catalysis is effective using aliphatic alcohols of

different length as substrates, a very interesting property in view of possible synthetic applications (Gil-Muñoz, 2015).

Therefore, previous studies on this fungal isolate indicated its potential interest for bioconversion of lignocellulosic biomass, from both the hydrolytic and synthetic perspectives. However, its hemicellulolytic capacity, and particularly the xylanolytic one, had not yet been specifically evaluated. The exploitation of that potential was the main goal of this doctoral thesis, which is based on the characterization of one β-xylosidase and one endoxylanase from *T. amestolkiae* CIB. The description of their properties together with the study of some applications, focusing on those related to obtaining xylosides of biotechnological interest and xylooligosaccharides, will be presented and discussed along the following chapters.

2. AIMS

Xylan is the most abundant hemicellulose and the second major carbon reservoir in the biosphere; therefore the industrial uses of lignocellulose require an effective exploitation of this polysaccharide. In this sense, filamentous fungi constitute a valuable source of hemicellulases active on xylan and xylooligosaccharides, whose catalytic properties can be applied for obtaining high value-added products. The aim of this thesis was the study and exploitation of the xylanolytic machinery from *T. amestolkiae*. With this purpose, the following tasks were established:

a) Study of the production by *T. amestolkiae* of enzymes implicated in xylan degradation.

b) Purification and characterization, from a biochemical and molecular perspective, of the main xylanolytic enzymes secreted by this fungus.

c) Study of potential biotechnological applications of these catalysts for the valorization of plant biomass.

3. MATERIALS AND METHODS

3.1. MATERIALS

Unless stated otherwise, chemicals and reagents were purchased from Sigma-Aldrich. Hydroxytyrosol (HT) and hydroxytyrosol acetate (HTA) were provided by Seprox Biotech. Xylobiose was from TCI Europe. Xylotriose, xylotetraose, xylopentaose, xylohexaose and Xylose Assay Kit were purchased from Megazyme. Glucose-TR Kit was provided by Spinreact. EnzChek® Ultra Xylanase Assay Kit was from Invitrogen. DNeasy Plant Mini Kit and QIAquick Gel Extration Kit were provided by Qiagen. Microcrystalline cellulose, disc filters, ultrafiltration membranes and Amicon® centrifugal devices were from Merck-Millipore. Bacto yeast extract and Bacto Peptone were purchased from BD Biosciences. Hydroquinone (HQ) was provided by Acros Organics. Catechol was purchased from Fisher Scientific. Resveratrol (RES) was from Seebio Biotechnology, Inc. Epigallocatechin gallate (EGCG) was provided from Zhejiang Yixin Pharmaceutical Co., Ltd.

3.2. GENERAL MICROBIOLOGICAL METHODS

3.2.1. Fungal strain and culture media

T. amestolkiae strain CIB was isolated from cereal waste and deposited at the IJFM (Instituto "Jaime Ferrán" de Microbiología) culture collection of the "Centro de Investigaciones Biológicas" (Madrid, Spain), with the reference A795.

Sporulation took place after culturing the fungus on 2% agar-malt Petri dishes at 26-28 ^{o}C for 7 days. About 1 cm^2 of agar-malt with growing mycelium was cut and added to a 5 mL solution of 1% NaCl and 0.1% Tween 80. The mixture was shaken and 200 µL were used to inoculate 250 mL flasks with 50 mL of CSS medium, containing (L^{-1}): 40 g glucose, 0.4 g $FeSO_4 \cdot 7H_2O$, 9 g $(NH_4)_2SO_4$, 4 g K_2HPO_4, 26.3 g corn steep solid, 7 g $CaCO_3$ and 2.8 mL soybean oil. pH was adjusted to 5.6 and the culture was incubated at 28 °C and 180 rpm for 5 days.

Enzyme production was carried out in 250 mL flasks with 50 mL of Mandels medium and 2 mL of the CSS culture prepared for inoculum. Mandels medium contained (L^{-1}): 2.0 g KH_2PO_4, 1.3 g $(NH_4)_2SO_4$, 0.3 g urea, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.3 g $CaCl_2$, 5 mg $FeSO_4 \cdot 7H_2O$, 1.6 mg $MnSO_4 \cdot H_2O$, 1.4 mg $ZnSO_4 \cdot 7H_2O$, and 1 g Bacto Peptone. Mandels medium was supplemented with 2% (w/v) beechwood xylan (≥90% xylose) as carbon source and endoxylanase and β-xylosidase inducer. Beechwood xylan is a hardwood xylan with a backbone of β-1,4-linked D-xylopyranosyl residues. Branches are mainly composed of 4-*O*-methylglucuronic acid attached to xylose C2 position and acetyl groups at

C2 or C3 positions (Freixo and De Pinho, 2002). In some assays 1% or 3% xylan, 1% D-xylose 1% D-glucose or 1% microcrystalline cellulose were used as alternative carbon sources. Cultures were incubated at 28 °C and 180 rpm, and samples were periodically withdrawn from three replicate flasks and centrifuged at 20,000 × *g* for 5 min to separate the culture liquids from the mycelium.

3.2.2. Yeast strain and culture media

Pichia pastoris strains KM71 and GS115 were purchased from Invitrogen. Selected clone (section 2.3) was first grown in yeast nitrogen base (YNB) agar plates. Yeast was then transferred to 250 mL flask with 50 mL of YEPS medium, containing (L^{-1}): 10 g Bacto yeast extract, 20 g Bacto Peptone, 10 g sorbitol, 2.403 g K_2HPO_4, 11.87 KH_2PO_4. pH was adjusted to 6 and cultures were incubated at 28 °C and 250 rpm overnight. Then, production was carried out in 1-L flasks with 100 mL of YEPS medium and 3.5 mL of the former preinoculum. Cultures were incubated at 28 °C and 250 rpm for 10 days with daily addition of 5 g/L methanol. Samples were periodically withdrawn to measure β-xylosidase activity and absorbance at 600 nm (OD_{600}) as estimation of yeast growth.

3.2.3. Screening for high-production yeast clones

P. pastoris transformed clones were screened for maximal production of β-xylosidase activity. The screening was performed on 25 clones from each strain. The colonies were cultured in a 96-well plate with 100 μL of YEPS medium per well. The medium included 5 g/L of methanol as inducer of gene expression. Cultures were incubated at 28 °C and 250 rpm and 50 μL of YEPS with 5 g/L of methanol were added at 24 and 48 h. Controls with both non-transformed *P. pastoris* KM71 and GS115 strains were included.

After 72 h the plate was centrifuged at 2,000 g for 15 min at 4 °C and 50 μL of each supernatant was placed into a new plate and incubated with 50 μL of a substrate solution containing 7 mM *p*-nitropheynl β-D-xylopyranoside (*p*NPX), 100 mM sodium formate buffer (pH 3) and 0.2% bovine serum albumin (BSA). The incubation was carried out at 50 °C for 10 min with gentle agitation. The reactions were stopped by the addition of 100 μL of 2% Na_2CO_3 and the release of 4-nitrophenol (*p*NP) was measured at 410 nm in a plate reader (SPECTRAMax Plus 384, Molecular Devices).

The two clones with highest activities from each strain were selected and liquid cultures were carried out as described in section 2.2 in order to compare clones in terms of secreted β-xylosidase activity.

3.3. PROTEIN PURIFICATION

Purification of the enzymes was performed chromatographically by using an ÄKTA Purifier chromatography system (GE Healthcare) and following the protocols described below.

3.3.1. Native enzymes purification

Production of native endoxylanase and β-xylosidase was carried out by culturing *T. amestolkiae* in 250 mL flasks with 50 mL of Mandels medium and 2% beechwood xylan as described above. 3-day-old cultures were harvested by filtering through filter paper in order to separate mycelium from culture liquids. The filtrate was centrifuged at 10,000 × *g* and 4 °C for 30 min and the supernatant filtered through 0.8, 0.45 and 0.22 μm disc filters. Finally, the crude was concentrated and dialyzed using a 5-kDa cutoff membrane. Dialysis was performed against 10 mM sodium acetate buffer (pH 4) or 10 mM sodium phosphate buffer (pH 6) for β-xylosidase and endoxylanase purification respectively.

β-xylosidase purification

The dialyzed crude enzyme was loaded onto a 5 mL-HiTrap SPFF cartridge (GE Healthcare), equilibrated in 10 mM sodium acetate buffer (pH 4). The bound proteins were eluted with a linear gradient of 1 M NaCl from 0 to 50% in 25 mL at a flow rate of 1 mL/min. The column was then washed with 1 M NaCl (10 mL), and allowed to re-equilibrate with the starting buffer for 10 min. Fractions with β-xylosidase activity were collected, desalted using PD-10 columns (GE Healthcare) equilibrated with 10 mM sodium acetate buffer (pH 4), and applied to a 1 mL Mono S 5/50 GL column (GE Healthcare) previously equilibrated in the same buffer. Proteins were eluted with a linear gradient of 1 M NaCl from 0 to 40% in 25 mL at a flow rate of 1 mL/min. The column was washed with 1 M NaCl (5 mL) and re-equilibrated to the starting conditions for 5 min. Fractions with β-xylosidase activity were collected, mixed, dialyzed and concentrated by ultrafiltration using Amicon® Ultra-15 centrifugal devices (5 kDa cutoff). Finally, samples were applied onto a Superose 12 HR 10/30 (GE Healthcare) equilibrated and eluted with the same buffer with 100 mM NaCl at a flow rate of 0.5 mL/min for 50 min. The purified enzyme was concentrated by ultrafiltration (5 kDa cutoff) and stored at 4 °C. The isolated β-xylosidase was named BxTW1.

Endoxylanase purification

One endoxylanase was isolated through two chromatographic steps. The dialyzed crude extract was loaded onto a 5 mL-HiTrap QFF cartridge (GE Healthcare) equilibrated in 10 mM phosphate buffer (pH 6) and a flow

rate of 1 mL/min. Protein elution was carried out by applying a linear gradient of 1 M NaCl from 0 to 50% for 25 min. Then, the mobile phase mix changed to 100% 1 M NaCl for 10 min and finally to 0% for 10 min in order to wash and re-equilibrate the column, respectively. Fractions displaying endoxylanase activity were collected, dialyzed in 10 mM phosphate buffer (pH 6) and concentrated by using 5 kDa Amicon® Ultra-15 centrifugal filter units. Concentrated samples were applied onto a Superdex™ 75 10/300 GL column equilibrated in 100 mM NaCl 10 mM phosphate (pH6) buffer and eluted at a flow rate of 0.5 mL/min for 60 min. The purified endoxylanase (XynM) was dialyzed and concentrated by ultrafiltration (5 kDa cutoff) and stored at 4 °C.

3.3.2. Recombinant β-xylosidase purification

For rBxTW1 purification, 10-day-old cultures of *P. pastoris* were harvested and centrifuged at 10,000 × *g* and 4 °C for 20 min. The supernatant was sequentially filtered through 0.8, 0.45 and 0.22 μm disc filters. Finally, the crude was concentrated and dialyzed against 10 mM acetate buffer (pH 4) using a 50-kDa cutoff membrane. Then, the chromatographic system was equilibrated in 10 mM sodium acetate buffer (pH 4) and the enzymatic crude was loaded onto a 5 mL-HiTrap SPFF cartridge. The elution of the bound proteins was carried out by applying a linear gradient of 1 M NaCl from 0 to 50% in 25 mL. The column was then washed with 1 M NaCl in 10 mL and re-equilibrated by applying 10 mL of the starting buffer. A flow rate of 1 mL/min was maintained during the entire process. Fractions with β-xylosidase activity were collected, pooled together, dialyzed and concentrated by ultrafiltration using 50 kDa cutoff Amicon Ultra-15 centrifugal devices. The purified enzyme was stored at 4 °C.

3.4. BASIC CHARACTERIZATION OF PURIFIED ENZYMES

Unless stated otherwise, 0.1% bovine serum albumin (BSA) was added to all assays. BSA is well-known for increasing enzyme stability in different environments and ensure the validity of the results regardless of the enzyme concentration, which otherwise would have a strong influence especially in the determination of thermal stabilities (Chang and Mahoney, 1995).

3.4.1. Enzyme and protein assays

Hydrolytic activity against glucose-containing substrates was measured by quantifying free glucose after the enzymatic reactions. The measure was carried out colorimetrically through the coupling of glucose

oxidase and peroxidase reactions using the Glucose-TR Kit following the manufacturer indications.

Proteins were quantified by the BCA method, using Pierce reagents and bovine serum albumin as standard, according to the manufacturer's instructions.

β-Xylosidases: specific assays

β-Xylosidase activity was measured spectrophotometrically by the release of *p*NP (ε_{410} = 15,200 M^{-1} cm^{-1}) from *p*NPX. The standard reaction mixture consisted of 3.5 mM *p*NPX, 50 mM sodium citrate or acetate buffer (pH 5) and the appropriate dilution of the purified enzyme or culture crude extract. Standard assays were incubated at 50 °C and 500 rpm for 5 and 10 min, in order to check the linearity of the measured activity, and the reactions were stopped by the addition of Na_2CO_3 at a final concentration of 135 mM. One unit of β-xylosidase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of *p*NPX per minute.

The measurement of the β-xylosidase activity with xylobiose, xylooligosaccharides and beechwood xylan was performed by direct quantification of the released xylose. It was carried out either by gas chromatography-mass spectrometry (GC-MS) or spectrophotometrically, the latter using standards and reagents of the D-Xylose Assay Kit and following the manufacturer indications.

Prior to GC-MS analysis, the reaction products were converted into volatile derivatives. To do so, the reaction mixture was dried and its components reduced with $NaBH_4$ at room temperature for 16 h and subsequently acetylated at 100 °C with pyridine and acetic anhydride according to Laine *et al*. (1972). Using this procedure, xylose was converted into its alditol acetate and *p*NP into its acetylated derivative. The samples were dried and resuspended in chloroform. Identification and quantification were performed by gas GC-MS on a 7890A-5975C instrument (Agilent), using a HP5MS fused silica column (30 m × 0.25 mm I.D. × 0.25 μm film thickness) with He as the carrier gas. Injector was set at 320 °C and auxiliary temperature to 280 °C. Samples (1 μL) were injected and analyzed with a temperature program: 175 °C for 1 min, then 2.5 °C min^{-1} to 215 °C, 10 °C min^{-1} to 225 °C, and finally 225 °C for 1.5 min. Identification was performed on the basis of the coincidence of the retention time of sample components with those previously measured for standards analyzed in identical conditions. Depending on the reactions, inositol or galactosamine were used as internal standard, to avoid overlapping with the reaction products.

The D-Xylose Assay Kit method is based on the complete conversion of free xylose into its beta anomer and then into D-xylonic acid, releasing

NADH. Xylose concentration is determined by following NADH absorbance at 340 nm.

Endoxylanase: specific assays

Endo-β-1,4-xylanase activity was determined by measuring the release of reducing sugars according to the Somogyi-Nelson method (Nelson, 1944). 200 µL-samples were incubated together with 200 µL Somogyi I/Somogyi II 4:1 (v/v) solution and incubated at 100 °C for 15 min. The solution was cooled at room temperature in a water bath for 5 min and then 200 µL Nelson reagent and 2.4 mL distilled water were added. Absorbance was measured at 540 nm and experimental data was converted into xylose equivalents using a calibration curve for this monosaccharide. One unit of endoxylanase activity was defined as the corresponding to the release of 1 µmol of reducing sugar per minute. The standard reaction mixture consisted of 2.5% beechwood xylan, 50 mM sodium acetate buffer (pH 5) and the appropriate dilution of the purified enzyme or culture crude extract. Standard assays were incubated at 50 °C and 1,200 rpm for 5 and 10 min, in order to check the linearity of the activity.

3.4.2. Molecular size and pI determination

The molecular mass of the purified enzymes was estimated by SDS-PAGE acrylamide gels (Wasay et al., 1998) using Precision Plus Protein™ Dual Color Standards (Bio-Rad) and proteins were stained with Coomassie Brilliant Blue R-250. Acrylamide content of the gels was 7.5% for β-xylosidases and 12% for endoxylanase.

The molecular mass of the purified proteins was also calculated from size exclusion chromatography on a Superose 12 HR 10/30 column, previously calibrated with a standard protein kit (GE Healthcare) containing chymotrypsinogen A (19.5 kDa), ovalbumin (48.2 kDa), BSA (73.5 kDa), aldolase (170 kDa) and ferritin (460 kDa). The accurate molecular mass and homogeneity of the pure enzymes was determined by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) (Autoflex III, Bruker Daltonics) externally calibrated with Bovine Albumin, covering the range from 30 to 150 kDa.

The isoelectric point (pI) of the proteins was determined by isoelectrofocusing, in 5% polyacrylamide gels using pH 3-10 ampholytes (GE Healthcare), with 1 M H_3PO_4 and 1 M NaOH as anode and cathode buffers, respectively. The pH gradient was measured directly on the gel using a contact electrode (Crison) and the obtained values were related to their positions in the gel in order to elaborate a calibration curve. Enzyme bands were detected by activity assays, carrying out the incubation of the gels with specific substrates and visualizing fluorescent products under UV light using the Gel Doc™ XR+ system (Bio-Rad) and pI was calculated

interpolating the migration distances in the calibration curve. β-Xylosidase substrate was *p*-methylumbelliferyl β-D-xylopyranoside and the gel was incubated in 20 mM sodium acetate buffer (pH 5) containing 40 μM of this xylosyl derivative for 0.5 h at 50 °C. The detection of β-xylosidase activity was based on the fluorescence of the released 4-methylumbelliferone. Visualization of endoxylanase required the use of 6,8-difluoro-4-methylumbelliferyl β-D-xylobioside, the main reagent of the EnzChek® Ultra Xylanase Assay Kit. The assay was carried out incubating the gel in 20 mM sodium acetate buffer (pH 4) containing 52.5 μM of the former substrate for 0.5 h at room temperature and the fluorescence of the released 6,8-difluoro-4-methylumbelliferone revealed the position of the enzyme.

The coding DNA sequence of BxTW1 (section 5.1) and the putative mature gene of XynM without introns (section 5.2) were used to predict the theoretical pI and molecular mass of both proteins. To do so, nucleotide sequences without introns were first translated to amino acids using the ExPASy Bioinformatic Resource Portal. These sequences were submitted to the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) to identify the signal peptides, which were excluded from the mass prediction. Then, the putative mature protein sequences were analyzed using the Compute pI/Mw tool of the ExPASy Bioinformatic Resource Portal.

3.4.3. Temperature and pH profiles

The optimal temperature of the enzymes was determined incubating these proteins in a range from 30 to 70 °C, for 5 and 10 min. The optimal pH was determined using a pH range from 2.2 to 9 at 50 °C for 10 min. In order to adjust pH values, the designated buffer of the standard mix reaction was substituted by the one appropriate for each segment of the range: glycinate (2.2-3), formate (3-4), acetate (4-5.5), phosphate (5.5-7).

Thermostability was evaluated by two approaches: T50 and an alternative assay using longer times of incubation. T50 is defined as the temperature at which the enzyme loses 50% activity after 10 min of incubation; it was determined heating the protein in a range of temperatures from 45 to 75 °C in 10 mM sodium acetate buffer (pH 4) for 10 min, cooling at 4 °C for 10 min and rewarming to room temperature for 5 min before measuring the residual activity by the respective standard assay. The temperature at which the enzyme retained the maximum residual activity was taken as 100%. The additional assay of thermotolerance was carried out by incubating the purified enzyme in 10 mM sodium acetate buffer (pH 4) for 72 h at temperatures varying from 30 to 70 °C. Samples were collected at different times and residual activity was assayed in standard conditions, assigning 100% value to the initial activity.

Regarding to pH stability, it was analyzed in the range from 2.2 to 9 incubating the samples at 4 °C for 72 h. As in the previous assay, 100% corresponds to the initial activity.

3.4.4. Effect of common chemical compounds

The effect of common chemical compounds on the activity of the enzymes from *T. amestolkiae* was studied. The list of the reagents tested included some or all the following compounds: LiCl, KCl, $AgNO_3$, $MgSO_4$, $CaCl_2$, $BaCl_2$, $MnCl_2$, $FeSO_4$, $CoCl_2$, $NiSO_4$, $CuSO_4$, $ZnSO_4$, $HgCl_2$, $Pb(NO_3)_2$, $AlNH_4(SO_4)_2$, $FeCl_3$ and ethylenediaminetetraacetic acid (EDTA), which were assayed at a final concentration of 5 mM in the reaction mix, and 2-mercaptoethanol (2-ME) and dithiothreitol (DTT) which were added at a final concentration of 10 mM. The assay was carried out in the reported standard conditions for each enzyme using sodium acetate buffer, although native BxTW1 was also assayed in 50 mM sodium citrate (pH 5), to test the effect of the mild chelating effect described for citrate (Wasay et al., 1998).

3.4.5. Evaluation of protein glycosylation

Protein sequence of native β-xylosidase was submitted to the Eukaryotic Linear Motif resource (http://elm.eu.org/) in order to search for predicted post-translational modification sites in the native enzyme. The following parameters were selected: Extracellular, as Cell Compartment; *T. amestolkiae* as Taxonomic Context; and 100 as Motif Probability Cutoff.

The *N*-carbohydrate content of native and recombinant β-xylosidases was demonstrated by the difference between the theoretical protein molecular mass and the molecular mass before and after treatment with endoglycosidase H (Endo H; Roche), with latter values estimated by SDS-PAGE in 7.5% polyacrylamide gels and accurately obtained by MALDI-TOF mass spectrometry.

Additionally, the glycosylation profile of rBxTW1, due to its potential effect on the enzyme activity was subjected to an in depth analysis and compared to BxTW1. To determine their monosaccharide composition, protein samples were first hydrolyzed with 3 M trifluoroacetic acid (TFA, 121 °C, 1 h), and derivatized and analyzed as reported by Bernabé *et al*. (2011). The linkage types in the glycan chains of the protein were determined after methylation analysis of dry samples (1-3 mg), dissolved in dimethyl sulfoxide and processed according to the method of Ciucanu and Kerek (1984). The per-*O*-methylated polysaccharides were hydrolyzed with 3 M TFA, derivatized to their corresponding partially methylated alditol acetates, and analyzed by gas chromatography-mass spectrometry as described elsewhere (Bernabé et al., 2011).

3.4.6. Substrate specificity

Specificity of β-xylosidases

The activity of pure β-xylosidases was tested against 3.5 mM *p*NPX, *p*-nitropheynl α-L-arabinopyranoside (*p*NP-Arap), *p*-nitropheynl α-L-arabinofuranoside (*p*NP-Araf), *p*-nitropheynl β-D-glucopyranoside, *o*-nitropheynl β-D-glucopyranoside, *p*-nitropheynl α-D-glucopyranoside, *p*-nitropheynl α-L-rhamnopyranoside, *p*-nitropheynl β-D-galactopyranoside, and *p*-nitropheynl β-D-fucopyranoside; 20 mM xylooligosaccharides from X2 to X6, lactose, maltose, sucrose, lactose, gentiobiose and cellobiose; and 20 g/L beechwood xylan and laminarin from *Laminaria digitata*. The assays were carried out at standard conditions and activity was measured by quantification of the released 4-nitrophenol in the case of nitrophenyl substrates, xylose for xylooligosaccharides, and glucose for disaccharides and laminarin.

Specificity of endoxylanase

The activity of pure endoxylanase was tested against 25 g/L beechwood xylan, 20 g/L carboxymethylcellulose, 10 g/L microcrystalline cellulose and 3.5 mM *p*NPX and *p*NPG. The assays were carried out at standard conditions and activity was measured by quantification of the released nitrophenol in the case of nitrophenyl substrates and total reducing sugars for beechwood xylan, microcrystalline cellulose and carboxymethylcellulose.

3.4.7. Kinetics determination

The experimental data derived from hydrolysis of the selected substrates were adjusted by least squares to the Lineweaver-Burk linear equation of the Michaelis-Menten model.

β-Xylosidase kinetics

Selected substrates for the kinetics determination of β-xylosidases were *p*NPX, *p*NP-Arap, *p*NP-Araf, xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose and beechwood xylan. The kinetic parameters were obtained by using increasing substrate concentrations in a range from 0.078 to 20 mM and from 0.625 to 40 g/L in the specific case of beechwood xylan. One unit of activity against XOS was defined as the amount of enzyme that totally hydrolyzes 1 μmol of the selected xylooligosaccharide to xylose per minute. Product inhibition by xylose was also studied and K_i determined against *p*NPX in the presence of 2.5, 5 and 10 mM xylose.

A deeper study of native BxTW1 behavior using xylotriose as substrate was carried out by NMR spectroscopy. The assay consisted on incubating 20 mM xylotriose with 800 mU/mL of BxTW1 (measured in standard conditions) in 50 mM formate buffer (pH 3) at 25 °C. The concentration of residual substrate and reaction products (disaccharide and monosaccharide) was followed by acquiring ^{1}H-NMR spectra on a Bruker 600 MHz spectrometer at different reaction times, until detecting the complete conversion of xylotriose and xylobiose in xylose. The amounts of each compound were compared integrating distinctive signals: xylose was analyzed from H5 (δ 3.8), and xylotriose from H'5 (δ 4.03). Xylobiose lacked a specific signal in the ^{1}H-NMR spectrum, so it was quantified by subtracting xylotriose H'5 (δ 4.03) from H5 (δ 3.9), which overlaps the signals from xylobiose and xylotriose.

Endoxylanase kinetics

The kinetics for hydrolysis of beechwood xylan by the endoxylanase was determined. K_m and V_{max} were calculated by measuring the enzymatic activity against a range of substrate concentrations from 1.5 to 70 g/L.

3.4.8. Reaction mechanism determination

The enzyme mechanism (retaining or inverting) of BxTW1 was determined by ^{1}H-NMR spectroscopy. The assay was carried out using 15 mM *p*NPX, 800 mU/mL of native β-xylosidase (measured in standard conditions) and 50 mM formate buffer (pH 3) at 25 °C. All reagents were prepared in deuterated water. Enzyme and substrate were mixed inside the NMR tube and the enzyme's mechanism was elucidated by determining the anomeric configuration of the newly formed D-xylose. ^{1}H-NMR 1D spectra, acquired with 32 scans on a Bruker 600 MHz spectrometer, were recorded at different times from 0 to 24 h.

3.4.9. Peptide mass fingerprinting and N-terminal determination

Peptide mass fingerprinting was performed from gel pieces of XynM and BxTW1 protein bands from Sypro-stained SDS-gels. Those were excised and digested following a protocol based on Shevchenko *et al.* (2006) with the minor variations reported by Russo *et al.* (2012). MALDI–MS and MS/MS data were combined through the BioTools 3.0 program (Bruker-Daltonics) to interrogate the NCBI non-redundant protein database using MASCOT software 2.3 (Matrix Science). Relevant search parameters were set: trypsin as enzyme, carbamidomethylation of cysteines as fixed modification, methionine oxidation as variable modification, 1 missed cleavage allowed, peptide tolerance of 50 ppm and MS/MS tolerance of 0.5 Da. Protein scores greater than 75 were considered significant (P<0.05).

The N-terminal amino acid sequence of XynM was analyzed by sequential Edman degradation in a Procise 494 protein sequencer (PerkinElmer). Homology searches were performed using the BLAST program to interrogate the NCBI non-redundant protein database.

3.4.10. Enzyme classification

The putative coding reading region of BxTW1 and XynM, without introns and signal peptide, was translated to protein and submitted to dbCAN server (Yin et al., 2012) to assign their respective glycosyl hydrolase family.

3.5. GENERAL MOLECULAR BIOLOGY METHODS

3.5.1. Primer design and amplification of BxTW1

To identify the gene coding for BxTW1, a BLASTP search against NCBInr using the peptides obtained by mass fingerprinting was carried out. The nucleotide sequences of the genes coding for β-xylosidases with high sequence identity to BxTW1 were retrieved from the database. Sequences were aligned using ClustalW and degenerate primers were designed in the conserved 5' and 3' regions, including ATG and Stop codons (BxTw1 Fw 5'- ATGGTYTACACCRYGCAATWYCTG -3' and BxTw1 Rv 5'- TYAMYTRKRATCAGGYTKAATCTCC -3'). The *bxtw1* gene was amplified by polymerase chain reaction (PCR) using genomic DNA as template. The DNA was extracted with DNeasy Plant Mini Kit, and PCR reactions contained 100 ng of DNA template, 1× PCR Buffer, 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 μM of each primer and 1 U of Taq DNA polymerase (Invitrogen) in a final volume of 50 μL. Reactions were subjected to an initial denaturation at 94 °C for 5 min, 34 cycles of amplification, each at 94 °C for 45 s, then 55 °C for 45 s and 72 °C for 2.5 min, followed by a final extension step at 72 °C for 5 min. Control reactions lacking template DNA were simultaneously performed.

The amplified sequences were separated in a 0.8% w/v agarose electrophoresis gel stained with GelRed, cut out, and purified by QIAquick Gel Extration Kit. PCR products were then inserted into pGEM-T easy cloning system (Promega) in order to transform the *Escherichia coli* DH5α strain. Clones containing the inserted fragments were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit, and the automated ABI Prism 3730 DNA sequencer.

3.5.2. Cloning of BxTW1 coding gene

Primers were designed based on the nucleotide sequence of the *bxtw1* gene coding for BxTW1 from *T. amestolkiae* previously sequenced

(GenBank accession no. KP119719), but excluding the region corresponding to the signal peptide, which was predicted by the SignalP 4.1 server.

Restriction sites for EcoRI and NotI were respectively added to the 5' and 3' primers (BxTw1 Fw, 5'-GAATTCCAGAACAACCAGACCTAT GCCAATTACTCC-3', and BxTw1 Rv, 5'- GCGGCCGCTTAATTGGGA TCAGGTTGAATCTCCTGCTC-3'). The *bxtw1* gene was amplified by PCR using the genomic DNA as template, since *bxtw1* has no introns. PCR mixtures contained 100 ng of DNA template, 1 μL PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 μM each primer, and 1 U of Expand™ High Fidelity PCR System DNA polymerase (Sigma-Aldrich) in a final volume of 50 μL. Reaction mixtures were denatured at 94 °C for 5 min and then subjected to 34 cycles of amplification, each at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 2.5 min, followed by a final extension step at 72 °C for 7 min. Control reactions lacking DNA template were simultaneously performed.

The PCR product, encoding BxTW1 without its signal peptide, was ligated to the yeast expression vector pPIC9 (Invitrogen), fused with the α-factor signal sequence and expressed under the transcriptional control of the methanol-inducible AOX1 promoter. The pPIC9:bxtw1 construct was named pPICW1N and used for transforming *P. pastoris* KM71 and GS115 after linearization with SacI (New England Biolabs). Transformed colonies were grown on YNB plates in the absence of histidine as selection marker.

3.5.3. Sequence prediction of XynM

The identification of the gene coding for XynM was performed based on the assembled genome of *T. amestolkiae* CIB (de Eugenio et al., 2017). A BLASTP search against the NCBInr database was carried out using the N-terminal and internal peptides sequences. The gene sequences of the best hits were used as queries to run a local BLASTN against the fungal genome. A predicted gene was identified and its sequence submitted to the SignalP 4.1 server for locating the signal peptide. An alignment between the gene and the best hits of the former BLAST search was performed for introns' identification. Local BLAST and alignments were performed on BioEdit sequence alignment editor (version 7.2.5).

3.6. TRANSXYLOSYLATION ASSAYS FOR (r)BxTW1

3.6.1. Effect of acceptor concentration

The relationship between initial rates of transxylosylation and acceptor concentration was studied. Xylobiose and xylotriose in a range of concentrations from 1.25 to 80 mM were incubated with 200 mU/mL of

BxTW1 (measured in standard conditions) and 50 mM formate buffer (pH 3) at 50 °C for 10 min. The reaction was stopped by incubation at 100 °C for 5 min. The presence and concentration of the remaining substrate, transxylosylation and hydrolysis products were determined by high-performance liquid chromatography (HPLC) on an Agilent 1200 series system equipped with a refractive index detector.

Aliquots of 100 µL were loaded onto a SUPELCOGEL™ C-G610H column (Sigma-Aldrich) equilibrated in 5 mM H_2SO_4 buffer. The column was previously calibrated injecting 100 µL of xylose and XOS samples, from X2 to X5, in a concentration range from 0.5 mM to 20 mM. From the area under the peaks, a calibration curve was calculated for each compound. Peaks were identified from their retention times, by comparison with those of the commercial standards, and their concentration calculated from the calibration curves. The results were used to estimate hydrolysis and transxylosylation ratios according to the equations below:

$$\text{Hydrolysis ratio (\%)} = \frac{\Delta[\text{Substrate}]_{0-\text{f}} - 2[\text{Xylosyl product}]_{\text{f}}}{\Delta[\text{Substrate}]_{0-\text{f}}} \times 100$$

$$\text{Transxylosylation ratio (\%)} = 100 - \text{Hydrolysis ratio (\%)}$$

3.6.2. Regioselectivity evaluation

Native BxTW1 regioselectivity when catalyzing the formation of a new glycosidic linkage was investigated. In order to obtain one or more transxylosylation products, 350 mM xylobiose was incubated in 50 mM formate buffer (pH 3) at 50 °C for 30 min, with 550 mU/mL of BxTW1. In a second experiment, 3.5 mM of *p*NPX was used as donor and 130 mM of xylose as acceptor, incubating with 500 mU/mL of BxTW1 in 50 mM formate buffer (pH 3), at 50 °C for 20 min. Both reactions were stopped by heating at 100 °C for 5 min. Samples were dried and resuspended in deuterated water. The identification of the transxylosylation products was accomplished by ^{1}H, ^{1}H-^{13}C HSQC, DOSY and DOSY-TOCSY-NMR on a Bruker 600 MHz spectrometer. The same approaches were applied separately to the commercial reagents added to each reaction, in order to discard signals from impurities and to confirm the assignations.

3.6.3. Evaluation of acceptor specificity

Several screenings were carried out in order to assay the range of valid acceptors for native and recombinant BxTW1. The evaluation included simple chemicals, specific groups of commercially valuable acceptors and highly diverse compounds.

Preliminary evaluation

The acceptor specificity of the enzyme against low complexity acceptors was evaluated using 3.5 mM *p*NPX as xylose donor, 24 mU/mL of BxTW1 (expressed in standard conditions) and incubating the reactions at 50 °C and 500 rpm for 4 h in 50 mM formate buffer (pH 3), with the presence of the tested acceptor. Assayed compounds and concentrations were as follows: 1 M methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, isobutanol or glycerol, or 70 g/L erythritol, mannitol, sorbitol, dulcitol, glucose, fructose, galactose, mannose, maltose, sucrose, trehalose or lactose. The selected acceptors were compared and grouped according to their physicochemical similarity after calculating the Tanimoto coefficient, using the workbench similarity tool from the ChemMine site (Backman et al., 2011). The reaction was stopped by incubation at 100 °C for 5 min. The amount of free *p*NP released from substrate hydrolysis was measured spectrophotometrically, while xylose content was determined by GC-MS, as described above. Transxylosylation, substrate hydrolysis and substrate consumption ratios were calculated from the concentration of xylose and *p*NP according to the equations below:

$$\text{Substrate consumption}(\%) = \frac{[p\text{NPX}]_0 - [p\text{NP}]_{\text{free}}}{[p\text{NP}]_0} \times 100$$

$$\text{Transxylosylation ratio}\ (\%) = \frac{[p\text{NP}]_{\text{free}} - [\text{xylose}]_{\text{free}}}{[p\text{NP}]_{\text{free}}} \times 100$$

$$\text{Hydrolysis ratio}\ (\%) = 100 - \text{Transxylosylation}\ (\%)$$

The results were presented as a heat map based on transxylosylation ratios. The hierarchical clustering analysis was performed using the clustergram algorithm within Matlab environment (MathWorks, Natick, MA).

Broad acceptor screening

A high-throughput screening was carried out in order to evaluate the potential of highly diverse compounds to act as acceptors in transxylosylation reactions catalyzed by rBxTW1. The assay was performed in accordance with Blanchard and Withers (2001) with minor variations. The enzyme was first inactivated as its 2-fluoroglucosyl species by incubating 150 µg/mL rBxTW1 in the presence of 5 mM 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-xylopyranoside, 5% dimethyl sulfoxide (DMSO), and 25 mM sodium phosphate (pH 6) in a final volume of 200 µL, at room temperature for 1 h. The sample was dialyzed by ultrafiltration with 10 kDa cutoff Viva spin 500 µL centrifugal filter units (Vivaproducts)

in order to remove the excess of inhibitor. The final concentration of inactivated enzyme was corroborated by absorption at 280 nm. An aliquot of the purified, inactivated enzyme was added to the wells of a 96-well plate together with each compound to be screened and the buffer. The final reaction mix was composed of 2.9 μg/mL inactivated rBxTW1, 25 mM sodium phosphate buffer (pH 6), 0.1% BSA and 20 mM or 40% saturation of the potential acceptor. pH 6 was selected instead of the standard pH 5, in order to directly follow the release of *p*NP with time. Controls of non-inactivated enzyme and inactivated enzyme without any potential acceptor were included in triplicate.

The plate was incubated at room temperature for 1 h. Then, *p*NPX was added at a final concentration of 1 mM and the continuous change in absorbance of each well was measured at 400 nm and 40 °C for 1 h in a plate reader (Molecular Devices Spectra Max 190 Reader). Compounds leading to higher rate of recovery from inhibition than the non-acceptor control were considered positive hits and potential acceptors for transxylosylation by rBxTW1.

A library of 87 compounds was screened in order to find potential transxylosylation acceptors for rBxTW1. The assayed compounds were as follows: methanol; ethanol; 1-propanol; 2-propanol; 1-butanol; 1-pentanol; 1-hexanol; cyclohexanol; 1-octanol; 2-mercaptoethanol, 2-methoxyethanol; 3-mercapto-1-propanol; 5-hexyne-1-ol; 1-ethynylcyclohexanol; 1-adamantanemethanol; 1-pyrenemethanol; 1,2-ethanediol; 1,3-propanediol; phenol; phenethyl alcohol; *o*-phenylphenol; *p*-phenylphenol; 4-(hexyloxy)phenol; *p*-methoxyphenol; *p*-vinylphenol; resorcinol; phloroglucinol; gallic acid; caffeic acid; 1-naphtol; 2-naphtol; DL-threitol; L-erythritol; L-arabitol; D-arabitol; D-sorbitol; D-galactitol; D-mannitol; myo-inositol; phenyl β-D-glucopyranoside; phenyl β-D-galactopyranoside; *p*-nitrophenyl α-D-xylopyranoside; *p*-nitrophenyl α-L-arabinopyranoside; *p*-nitropheynl α-D-galactopyranoside; *p*-nitrophenyl α-D-mannopyranoside;; *p*NPG; *p*-nitropheynl β-D-galactopyranoside; *p*-nitrophenyl β-D-mannopyranoside; *p*-nitrophenyl β-D-fucopyranoside; *p*-nitrophenyl β-D-glucuronide; *p*-nitrophenyl β-D-cellobioside; *p*-nitrophenyl β-D-lactopyranoside; 4-methylumbilliferyl β-D-xylopyranoside; 4-methylumbelliferyl β-D-glucopyranoside; 4-methylumbelliferyl β-D-galactopyranoside; 4-methylumbelliferyl β-D-cellobiopyranoside; D-xylose; L-arabinose; D-lyxose; D-ribose; D-glucose; D-glucal; D-galactose; D-galactal; D-mannose; 1,5-anhydro-D-glucitol; D-tagatose; D-allose; L-sorbose; L-rhamnose; L-fucose; D-fructose; sucrose; D-maltose; α-lactose; α,α-trehalose; D-cellobiose; gentiobiose; maltotriose; D-raffinose; L-serine; L-threonine; L-tyrosine; L-asparagine; L-arginine; L-cysteine. Stock solutions of these compounds were made in water to a final concentration, where possible, of 100 mM; some of these solutions were saturated.

Plant polyphenolic antioxidants

A screening using specifically plant phenolic antioxidants was performed. Transxylosylation reactions were carried out using 0.5 and 5 U/mL of rBxTW1 in the presence of 0.1% BSA and 50 mM sodium formate buffer (pH 3.0). 40 mM xylobiose (11.3 g/L) was added as donor and the selected antioxidants were tested as acceptors. Blank reactions with no enzyme were also assayed. Resveratrol, quercetin, (±)-α-tocopherol, hesperetin and hesperidin are highly hydrophobic acceptors so the addition of a co-solvent was necessary. These compounds were assayed at 10 g/L in 20% (v/v) acetonitrile and at 40 g/L in 50% (v/v) ethyl acetate. The moderately hydrophillic HT, gallic acid, L-ascorbic acid, ECGC, ferulic acid, HQ and catechol were assayed at 10 g/L without organic co-solvents. In the case of ECGC a concentration of 40 g/L without co-solvent was also tested. Reactions were carried out at 50 °C and 1,200 rpm with a final volume of 200 μL.

Samples (2 μL) were collected at 1 min, 30 min, 1 h, 2 h, 6 h, 24 h, 48 h and 72 h and reactions were followed by thin layer chromatography (TLC) in silica gel G/UV254 polyester sheets (0.2 mm thickness and 40 x 80 mm plate size) purchased from Macherey-Nagel, using ethyl acetate/methanol/H_2O 10:2:1 (v/v) as running buffer. Detection was performed under 254 nm UV light, and the pattern of spots in each reaction and its no-enzyme control were compared.

Acceptors identified as positive hits by TLC were subsequently analyzed by mass spectrometry. Samples were analyzed on a mass spectrometer with hybrid QTOF analyzer (model QSTAR, Pulsar I, from AB Sciex). Reaction samples were analyzed by direct infusion and ionized by electrospray (ESI-MS) with methanol as ionizing phase in positive reflector mode.

2,6-Dihydroxynaphthalene (2,6-DHN)

The role of 2,6-DHN as a transxylosylation acceptor for rBxTW1 was assayed. The compound was added to the reaction at a final concentration of 3 g/L together with 0.01 g/L rBxTW1, 20 mM xylobiose, 0.1% BSA, 50 mM sodium acetate buffer (pH 5) and 5% acetonitrile. The reaction was incubated at 50 °C and 1,200 rpm for 1 h. Samples were withdrawn at 10 min, 30 min and 1 h. The synthesis of new products was followed by TLC as described above.

3.6.4. Enzymatic synthesis of non-natural xylosides

The most relevant positive acceptors of section 3.6.3 were submitted to further assays in order to increase the production yields. Reaction parameters were adjusted by different approaches depending on the selected acceptor.

Enzymatic synthesis of phenolic xylosides

Reaction optimization with HT or HQ as acceptors and xylobiose as donor was assessed. A range of donor and acceptor concentrations, as well as different reaction times, were tested. The reactions were carried out in 50 mM sodium formate buffer (pH 3.0), 50 °C and 1,200 rpm. Table 3.1 summarizes the assayed conditions.

Table 3.1. Reaction conditions tested in order to study the kinetics of the transxylosylation reactions.

Acceptor	BxTW1 (U/mL)	[Acceptor] (mM)	[Donor] (mM)	Reaction time (min)
Hydroxytyrosol	18	75, 150, 600	40	60, 120
		300	40	10, 30, 60, 80, 120, 240
			20, 40, 80, 160, 350	60, 120
Hydroquinone	0.9	55, 220, 440	40	60, 120
		110	40	10, 20, 40, 60, 120, 240
			10, 20, 40, 80, 160, 350	60, 120

At the selected time intervals, reactions were stopped by enzyme denaturation at 100 °C for 5 min and stored at 4 °C. The concentration of the new xylosides was determined by HPLC. Concentration data, in terms of molarity, were used to calculate reaction yields according to the following equation:

$$\text{Yield (\%)} = \frac{[\text{Product}]}{[\text{Acceptor}]} \times 100$$

Reactions were performed in triplicate. Standard deviations were in all cases lower than 5%.

Enzymatic synthesis of xylosides from 2,6-dihydroxynaphthalene. Optimization by RSM

Optimization of the reaction conditions for the production of 2-(6-hydroxynaphthyl) β-D-xylopyranoside was attempted by using the response surface methodology. Design-Expert® software version 10.0.1.0 (Stat-Ease Inc) was selected for generating a Box-Behnken design matrix and for the analysis of generated data. Concentration of xylobiose (donor), 2,6-DHN (acceptor) and enzyme, reaction time, temperature and pH were selected as

the most significant parameters for xyloside production and included as independent variables for the development of the experimental design.

In this approach the parameters are studied at three levels: low, middle and high, leading to optimal values with a smaller number of designed experiments. The software then generates a polynomial quadratic equation from the obtained data which analyzes the effect of the independent variables on the response (Batra et al., 2014; Ma et al., 2009).

The variables and levels assayed are displayed in Table 4.7. Maximum and minimum levels were previously determined by using one factor at a time approach (data not shown).

3.6.5. HPLC analysis on synthesized xylosides

HPLC approach was selected to monitor the synthesis of the xyloside products and evaluate reaction yields. Once studies of optimization achieve the conditions for maximal production, the products of interest were also purified by HPLC.

Xylosides from hydroxytyrosol and hydroquinone

HPLC analyses were performed on an instrument equipped with a semipreparative HPLC quaternary pump 600E (from Waters Corporation), an autosampler ProStar (Agilent Technologies) and a photodiode-array detector (Agilent Technologies) controlled by software Varian LC Workstation 6.41. Reaction products were detected by their absorbance at 241 and 268 nm. The data recorded at 268 nm were used for quantification. The temperature of the column was kept constant at 40 °C. An ACE 3 C18-PFP column (15 x 4.6 mm, 5 µm, Advanced Chromatography Technologies Ltd.) was employed. The mobile phase was methanol/H_2O 20:80 v/v (both containing 0.1% acetic acid) at 0.7 mL/min for 12 min in the case of samples with HT as acceptor. Methanol/H_2O 5:95 v/v (both containing 0.1% acetic acid) at 0.7 mL/min for 15 min was selected in the case of assays with HQ. The quantification was carried out using the Varian Star LC Workstation 6.41. Calibration curves of HT and HQ standards were employed for the quantification of HT- and HQ-xylosides, respectively.

In order to identify the HT-xyloside (HTX) and perform assays of biological activity the reaction volume was increased up to 4 mL in the conditions of highest production: 350 mM xylobiose, 300 mM HT, 50 mM sodium formate buffer (pH 3.0) at 50 °C and 1,200 rpm for 2 h. HTX was purified by semipreparative HPLC. The 4 mL reaction mixture was loaded onto the semipreparative column ACE 5 C18-PFP (15 x 10 mm, 5 µm, Advanced Chromatography Technologies Ltd.). The mobile phase was methanol/H_2O 20:80 v/v (both containing 0.1% acetic acid) at 5.5 mL/min for 12 min. A three-way flow splitter (Dionex) was used. The fractions

containing the product were pooled and the solvent evaporated. The product was stored at -20 °C.

Xylosides from 2,6-dihydroxynaphthalene

HPLC analyses were performed on an Agilent 1200 series LC instrument equipped with a ZORBAX Eclipse XDB-C18 column (Agilent). The system was equilibrated in acetonitrile/H_2O 10:90 v/v (both containing 0.1% acetic acid) with a flow of 2 mL/min. To elute the reaction products, acetonitrile concentration increased along a linear gradient from 10 to 20% for 8 min. Then, the mobile phase mix changed to 95% acetonitrile for 3 min and finally to 10% for 3 min in order to respectively wash and re-equilibrate the column. The product peaks were detected by monitoring absorbance at 220 nm from the naphthalene ring and quantification was based on the areas under the peaks. As the reaction products are not commercially available, they were quantified from a calibration curve of 2,6-DHN.

With the purpose of identifying the obtained products, the reaction was scaled-up to 10 mL in conditions of high production predicted by the multiparametric model: 3 g/L 2,6-DHN, 50 mM xylobiose, 0.15 g/L rBxTW1 50 mM sodium acetate buffer (pH 5.5) and 39.5 °C for 80 min. The reaction was carried out at 1,200 rpm and stopped by heating at 100 °C for 5 min. After thermal shock, it was concentrated by speed vacuum before being loaded onto a semi-preparative column (Mediterranea sea_{18} TR-010006, Teknokroma) in order to be purified by HPLC. The system was equilibrated in acetonitrile/H_2O 10:85 v/v (both containing 0.1% acetic acid) and a flow rate of 2 mL/min was maintained during the entire process. The products were eluted by applying a gradient from 15 to 20% acetonitrile for 13 min. Then the concentration of acetonitrile was raised up to 95% for 3 min in order to wash the column. Finally, the system was allowed to re-equilibrate in the starting conditions for 4 min.

After collection and solvent evaporation, products were stored at -20 °C.

3.6.6. Identification of purified xylosides by Nuclear Magnetic Resonance

Once purified, the products synthesized by the catalytic action of rBxTW1 were redissolved in deuterated water and submitted to NMR analyses in order to determine its identity.

Identification of hydroxytyrosol xyloside

The structure of the derivative was determined using a combination of 1D (^{1}H, 1D-selective NOESY experiments) and 2D (COSY, DEPT-HSQC, NOESY) NMR techniques. The spectra of the sample, dissolved in

deuterated water (*ca.* 10 mM), were recorded on a Bruker AV-III 800 spectrometer equipped with a TCI cryoprobe with gradients in the Z axis, at a temperature of 298 K. Chemical shifts were expressed in parts per million with respect to the 0 ppm point of DSS (4-dimethyl-4-silapentane-1-sulfonic acid), used as internal standard. All the pulse sequences used were provided by Bruker. For the DEPT-HSQC experiment, values of 7 ppm and 2K points, for the 1H dimension, and 160 ppm and 256 points for the ^{13}C dimension were used. For the homonuclear experiments COSY and NOESY, 7 ppm windows were used with a 2K x 256 point matrix. For the NOESY and 1D-selective NOESY experiments, mixing times of 500-600 ms were used.

Identification of 2,6-dihydroxynaphthalene xylosides

Data were acquired at 308 K, using a Bruker AVANCE 500 MHz spectrometer. 1D ^{1}H NMR spectra, 2D homonuclear TOCSY (60 ms mixing time), and 2D heteronuclear ^{1}H-^{13}C HSQC experiments were acquired, in order to assign all NMR signals. For 1D ^{1}H, TOCSY, and ^{1}H-^{13}C HSQC, the standard zg, zgesp, dipsi2phpr, and hsqcedetgp pulse sequences implemented in TOPSPIN 2.1 acquisition software (Bruker) were employed.

3.6.7. Biological evaluation of HTX

The purified HTX was assayed in order to determine its potential as an anti-inflammatory and neuroprotective compound. These analyses also included HT, together with its lipophilic derivative HTA and the well-characterized antioxidant RES with a comparative purpose. The assays were carried out *in vitro* in cell cultures. SH-S5Y5 neurons were cultured in collagen-pretreated Petri-dishes with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 medium supplemented with penicillin/streptomycin and 10% inactivated fetal bovine serum (iFBS). RAW 264.7 macrophages were cultured in DMEM high glucose medium supplemented with penicillin/streptomycin and 10% iFBS.

To carry out all the assays described below, the tested compounds were dissolved in DMSO and added at different concentrations (1, 10 and 100 µM) to the cell cultures. The final DMSO percentage in each cell was adjusted to 1%. Averages and standard deviations of at least eight different readings from various experiments were calculated.

The cytotoxicity of the tested compounds to macrophages and neurons was evaluated before conducting the anti-inflammation and neuroprotection assays. Neuron assays were done in collagen-pretreated 96-well plates by seeding 2 x 10^4 neurons per well in a 100-µL volume, incubating for 24 h before compound addition. Macrophage assays were done in 96-well plates by seeding 2.5 x 10^4 macrophages per well in a 100-

µL volume and incubating for 4 h before compound addition. Cell viability was evaluated 24 h after compound addition by the mitochondrial MTT assay according to the manufacturer.

The anti-inflammatory effect was tested on macrophages that were cultured and plated as described for the cell viability assay. After 10-min incubation of the cells with solutions of the tested compounds in DMSO, 100 ng/mL of LPS were added in order to induce inflammation. Cell viability was evaluated 24 h after compound addition by the mitochondrial MTT assay.

To determine the neuroprotective effect, neurons were also cultured and plated as described in the cell viability assay. The compounds were added at the three concentrations tested and incubated for 10 min, before adding 100 µM hydrogen peroxide. Cell viability was evaluated 24 h after compound addition by mitochondrial MTT assay. Neuron recovery was calculated by normalizing the results from H_2O_2-neuron viability to the H_2O_2 positive and negative controls according to the equations displayed below:

$$\text{Normalization} = \frac{\text{X} - \text{Z}}{\text{Y} - \text{Z}} \times 100$$

$$\text{X} = \text{ Viability (\%) Compound} + \text{DMSO} + \text{H}_2\text{O}_2$$
$$\text{Y} = \text{ Viability (\%) DMSO}$$
$$\text{Z} = \text{ Viability (\%) DMSO} + \text{H}_2\text{O}_2$$

The data for the tested compounds are displayed in a 0-100% scale, where the H_2O_2 positive control (Z) is taken as 0% and the H_2O_2 negative control as 100%. Values between 0 and 100% indicate that the compound is partially neuroprotective while 100% or above mean complete neuroprotection.

3.7. PRODUCTION OF XYLOOLIGOSACCHARIDES BY XynM AND EVALUATION OF PREBIOTIC EFFECT

3.7.1. Production of XOS by enzymatic hydrolysis of birchwood xylan

The hydrolysis reactions contained 20 g/L birchwood xylan in 10 mM sodium acetate buffer pH 4.6 with 1 U/mL XynM. The reaction mixture (5 mL) was incubated at 50 °C and 600 rpm, for 96 h. 100 µL-aliquots of reaction solution and control, at different time points, were mixed with pure ethanol (final ethanol concentration 70% v/v) to inactivate the enzyme and precipitate the remaining xylan. The inactivated samples were centrifuged at 1,000 × *g* in order to analyze the supernatant by High-

Performance Anion-Exchange Chromatography Coupled with Pulsed Electrochemical Detection (HPAEC-PAD).

The analysis was carried out in an ICS3000 Dionex system consisting on a SP gradient pump, an AS-HV autosampler and an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode. An anion-exchange 3 × 250 mm Carbo-Pack PA-200 column (Dionex) was used at 30 °C. Eluent preparation was performed with Milli-Q H_2O. The initial mobile phase was 15 mM NaOH at 0.5 mL/min for 12 min. An 8 min-gradient from 15 mM to 75 mM NaOH and from 0 mM to 80 mM sodium acetate was applied. Then, the mobile phase composition varied from 75 mM to 100 mM NaOH and from 80 mM to 320 mM sodium acetate for 10 min. A final 15 min-gradient was programmed to return to the initial conditions (15 mM NaOH and 0 mM sodium acetate). The peaks were analyzed using the Chromeleon software. The flow rate was constant at 0.5 mL/min and 25 µL of each sample were injected. The identification and quantification of xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose was done using commercial standards. All samples were previously diluted 1:10 with H_2O and filtrated through 0.45 µm nylon filters.

3.7.2. Characterization of products by mass spectrometry

The molecular weight of hydrolysis products from birchwood xylan was assessed using a mass spectrometer with hybrid QTOF analyzer (model QSTAR, Pulsar I) (AB Sciex). Samples were analyzed by direct infusion and ionized by electrospray with methanol as ionizing phase both in positive and negative reflector modes.

3.7.3. Prebiotic effect determination of XOS from birchwood xylan

The biological effect of the obtained XOS was evaluated by carrying out fecal fermentation with this mixture. The profile of organic acids and the developed microbiome in the fermentations were determined in order to assess the prebiotic potential of birchwood XOS.

Fecal fermentations

Assays were carried out by triplicate in 10 mL microfermentors (24 multi-well plates; µ-24 Bioreactor) (Pall Corporation). The fresh fecal sample came from a female breast-fed baby who had not received antibiotic treatment for at least 3 months prior to experimentation and had no history of bowel disorders. Stools were vacuum stored and refrigerated until fermentation took place. Fresh feces (800 mg) were weighted and dissolved in the culture medium described by Macfarlane *et al*. (1998) in a proportion 1:5 (feces:medium) to hydrate them. Each microfermentor was inoculated with 500 µL of the homogenized mixture (fecal slurry). Before starting

fermentation, the headspace was displaced with nitrogen, and the whole assay was performed in anaerobiosis, maintaining temperature (37 °C) and pH (pH 5.5). A sample was taken from the initial fecal slurry as the time 0 control, and fermentations were harvested after 24 h and immediately frozen and stored at -20 °C.

Production of short-chain fatty acids (SCFAs) and other organic acids.

The content of acetic, propionic, butyric, lactic and succinic acid in the slurries from fermentations was evaluated by HPLC. For organic acid quantification, 0.2 mL of the fermented samples were centrifuged at 20,000 × *g* for 60 min, filtered by 0.45 µm pore size filters and diluted by 1:2 in MilliQ quality water. An aliquot of 20 µL of processed samples was analyzed using a HPLC Acquity equipped with a 300 × 7.8 mm Aminex HPX-87H column (Bio-Rad) and isocratically analyzed with 5 mM H_2SO_4 as eluent (0.6 mL/min flow rate). Peaks were detected with a refractive index detector.

Microbiome analysis

DNA from fecal fermentation samples was isolated according to Yuan *et al*. (2012) with minor modifications, with the aid of the MagnaPure Compact System (Roche Life Science), to avoid bias in DNA purification toward misrepresentation of Gram positive bacteria. For massive sequencing, the hypervariable region V3-V4 of bacterial 16S rRNA gene was amplified using key-tagged eubacterial primers (Klindworth et al., 2013) and sequenced with a MiSeq Illumina Platform, following the Illumina recommendations for Library preparation and sequencing for metagenomics studies.

The resulting sequences were split taken into account the barcode introduced during the PCR reaction. Quality control of the sequences was performed in different steps: i) quality filtering (minimum threshold of Q20) was performed using FASTX-Toolkit version 0.013, ii) primer (16S rRNA primers) trimming and length selection (reads over 300 nt) was done with cutadapt version 1.2. These FASTQ files were converted to FASTA files and UCHIME program version 7.0.1001 was used in order to remove chimeras that could arise during the amplification and sequencing step. Those clean FASTA files were subjected to analysis with QIIME version 1.8 with the parameters by default and SILVA 16s rRNA database version 123 in order to annotate each sequence at different phylogenetic levels (phylum, family and genus). Putative species level was afterword annotated comparing the taxonomical association found in QIIME database with NCBI database species annotation. Alpha diversity and beta diversity was conducted with QIIME.

The identity of *Staphylococcus hominis* was confirmed by PCR analysis using primers specifically designed for the detection of *Staphylococcus* species. A target region of *rpoB* and *tuf* genes was amplified according to primer design and PCR conditions reported elsewhere (Drancourt and Raoult, 2002; Martineau et al., 2001).

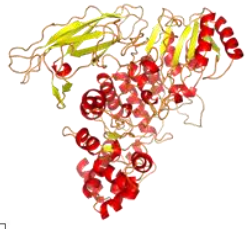
DNA from fecal fermentation was used as template in a final concentration of 5 ng/µL. The amplified PCR products were purified, sequenced and used to carry out a BLASTN search against the NCBInr database in order to establish *Staphyloccocus* sp identity.

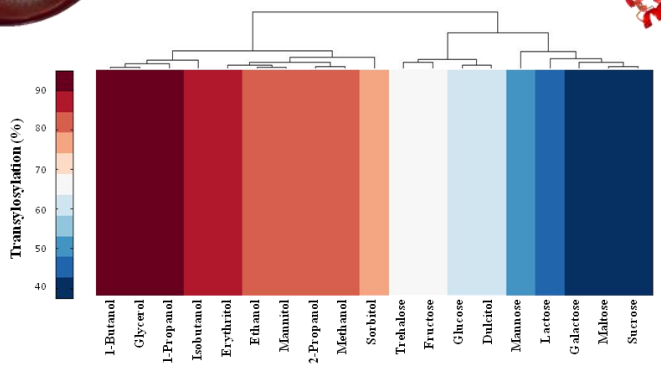
Microbiomes were grouped by treatment (control 0 h, control 24 h, 200 g/L XOS and 400 g/L XOS) and means were compared in order to determine if XOS displayed a demonstrable prebiotic effect in the tested conditions.

4. RESULTS AND DISCUSSION

4.1. CHAPTER I

CHARACTERIZATION OF A NOVEL pH-STABLE GH3 β-XYLOSIDASE FROM *Talaromyces amestolkiae*: AN ENZYME DISPLAYING REGIOSELECTIVE TRANSXYLOSYLATION





4.1.1. Background

The identification and characterization of β-xylosidases are currently outstanding topics. The needs for biomass exploitation in order to obtain goods from renewable sources make these enzymes very interesting from a biotechnological perspective. In addition, some retaining β-xylosidases are capable of catalyzing the formation of a new glycosidic linkage, transferring a xylosyl residue from a donor to an alcohol group of a particular acceptor in a process called transxylosylation (Fig. 4.1). This type of activity is especially interesting because this mechanism allows the synthesis of conventional as well as new xylooligosaccharides of different degrees of polymerization (DP) with a potential outlet in prebiotics and interest for pharmacological applications (Carvalho et al., 2013). As an example, novel glycosidic-polyphenolic antioxidants with greater solubility and bioavailability can be synthesized in such reactions (Chavez et al., 2006).

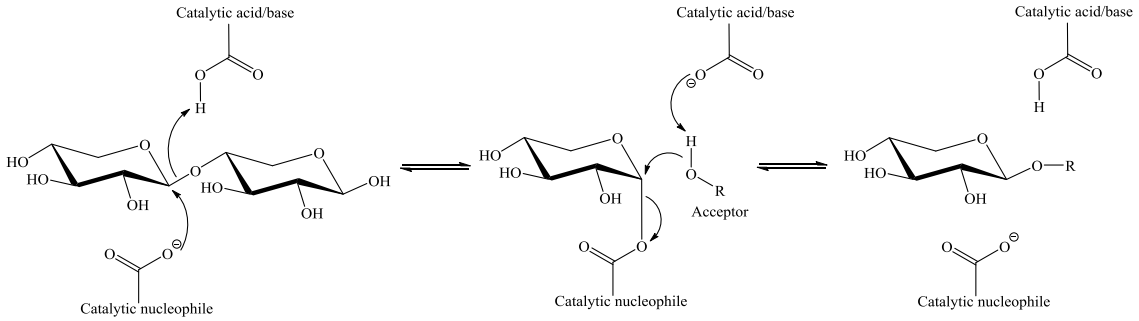


Fig. 4.1. Transxylosylation reaction catalyzed by a retaining β-xylosidase.

Many ascomycetes producing β-xylosidases and other xylanolytic enzymes have been described. Although *Aspergillus* and *Trichoderma* have been the most extensively studied, *Penicillium* strains seem to be good candidates as sources of lignocellulolytic enzymes (Chavez et al., 2006). In a previous study, a perfect state (determined when fungal sexual phase is observed) of a *Penicillium* species, identified as *T. amestolkiae*, was selected for secreting a large amount of cellulases and hemicellulases (Gil-Muñoz, 2015).

This chapter reports the production, isolation and biochemical characterization of a β-xylosidase from *T. amestolkiae*. In addition, the sequencing and molecular characterization of the new enzyme are presented and its potential interest in hydrolysis and regioselective transxylosylation reactions is discussed.

4.1.2. β-xylosidase production

The secreted proteins and β-xylosidase activity of *T. amestolkiae* cultures were studied in Mandels medium with different carbon sources. Figure 4.2A shows the β-xylosidase-inducer effect of 1% Avicel, 1% D-xylose or 1%, 2% or 3% beechwood xylan during 6 days. A control culture with 1% D-glucose as carbon source, which inhibits xylanases production by carbon catabolite repression (Knob et al., 2010), was also tested. The highest β-xylosidase activity was detected when 2% beechwood xylan was used as inductor, and the profile of total secreted proteins was similar to those detected with 3% xylan. Xylose addition also produced β-xylosidase release, although at a lower level. Figure 4.2B depicts the levels of secreted proteins during the culture time, showing a sharp increase of extracellular proteins at the end of the period. At this point, a very fragmented mycelium was observed under the light microscope (data not shown), probably related to cellular lysis and massive release of intracellular proteins. Based on these results, 2% beechwood xylan was chosen as the best inducer for β-xylosidase, which was produced, purified and characterized from these crudes.

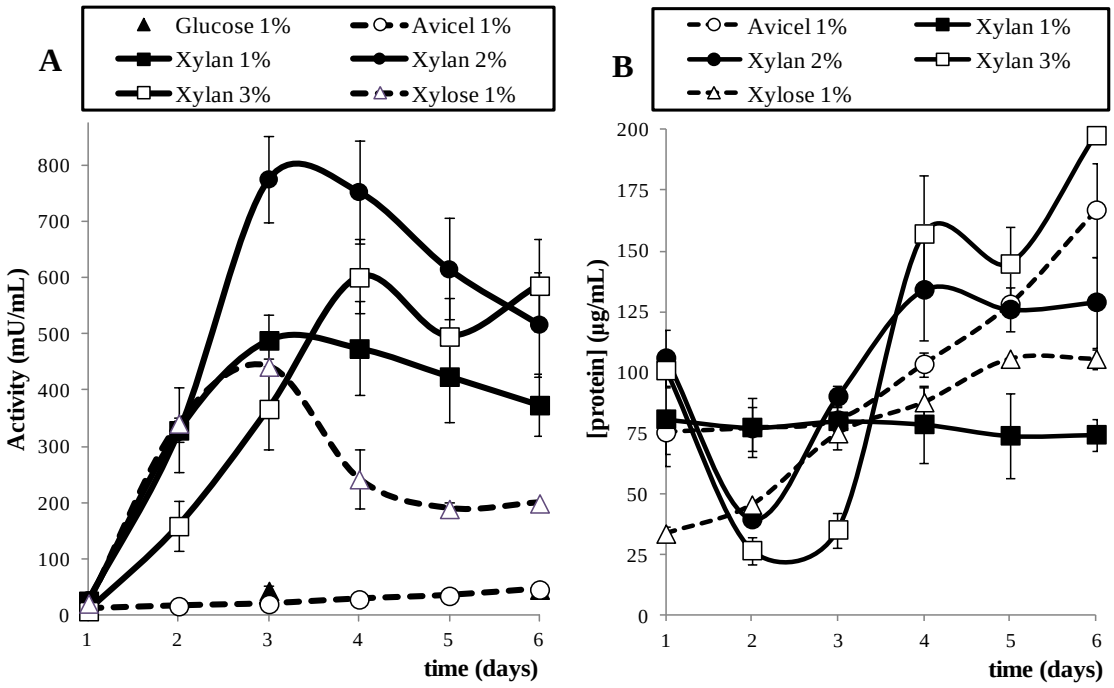


Fig. 4.2. Extracellular β-xylosidase activity (A) and protein concentration (B) of T. amestolkiae cultures in Mandels medium in the presence of different carbon sources.

Additional data on the production of β-xylosidase enzymes by the fungus were obtained by secretomic analyses, which were focused on the most intense spot of the 2D-gel. It was cut and identified by fingerprinting, showing that the highest identity match (90%) was XylA (gi:242771939), a

putative β-xylosidase from *T. stipitatus* that had not been previously characterized (Fig. 4.3).

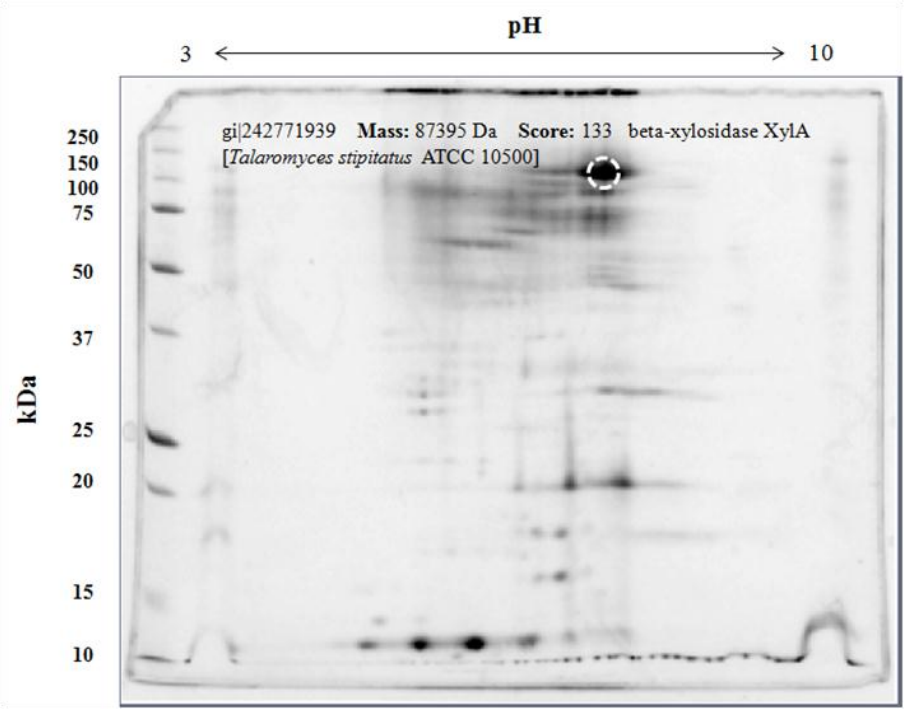


Fig. 4.3. 2D-gel from *T. amestolkiae* secretome grown in Mandels medium with 2% beechwood xylan. Selected spot marked in a white dashed circle was subsequently cut, digested and its highest identity match was identified by peptide fingerprinting as XylA protein from *T. stipitatus*.

The β-xylosidase levels released by *T. amestolkiae* in liquid cultures are in agreement with previous results described for *Aspergillus* and *Fusarium* strains when beechwood xylan was added as carbon source (Lenartovicz et al., 2003; Saha, 2001), and higher than those reported for other *Penicillium* species (Jørgensen et al., 2005; Knob and Carmona, 2011). Indeed, secretomic analyses indicated that BxTW1 is one of the most abundant proteins found in the fungal cultures under the conditions selected for maximal production. Although pure commercial xylan is not suitable for high-scale enzyme production, it has been established as the most used carbon source for research and the best inducer of xylanolytic enzymes (Milagres and Prade, 1994). As in other fungi, xylose acts as a weak inducer of β-xylosidase production in *T. amestolkiae* (Jørgensen et al., 2004), but glucose did not induce β-xylosidase production (Jørgensen et al., 2004; Terrasan et al., 2010).

4.1.3. Purification of β-xylosidase

Maximal β-xylosidase activity levels (800 mU/mL) were detected in 3-day-old cultures. Then, these crudes were collected for enzyme purification.

The first cation exchange chromatography step allowed the separation of a unique peak with β-xylosidase activity, eluting around 0.25 M NaCl, from most of the crude proteins. The peak with β-xylosidase activity was subsequently separated on Mono S 5/50, a high-resolution cation exchange column. β-xylosidase activity mainly eluted in three successive peaks between 0.20 and 0.25 M NaCl. A last size-exclusion step using a Superose 12 column was necessary for the complete purification of the protein, denominated BxTW1. The enzyme, dialyzed and concentrated, was stored at 4 ºC, remaining stable during at least six months.

The purification resulted in a final yield of 10.8% recovered activity. During the process, the specific activity increased from 1.0 to 47.1 U/mg, which implies a degree of purification of 46.6.

4.1.4. Physicochemical properties

The molecular mass of BxTW1, estimated from size exclusion chromatography, was around 200 kDa. However, analysis of the BxTW1 sequence (GenBank ID: KP119719) using the ExPASy Bioinformatics Resource Portal resulted in a theoretical molecular mass of 84,373.96 Da. SDS-PAGE of non-deglycosylated BxTW1 showed three bands of approximately 100 kDa (Fig. 4.4A), which is close to the value from MALDI-TOF-MS (Fig. 4.4B). The MALDI-TOF spectrum displayed the typical profile of a glycosylated protein, with a wide peak due to glycosylation heterogeneity. The technique allowed determining the accurate mass of one of the glycosylated isoforms (102,275 kDa) but the global enzyme mass could only be estimated on average around 100 kDa. The closeness of the peaks in the mass spectrum apparently corresponded to different glycosylation forms, which would be consistent with the identification of three separated peaks of β-xylosidase activity during high-resolution cation exchange chromatography. The peptide mass fingerprint of each one of the three bands was obtained, resulting in exactly the same fragmentation patterns (not shown). To discern if these molecular weight changes could be due to glycosidic content variations, the mature protein sequence was used to search for predicted post-translation modifications at ELM server, and 14 motifs for *N*-glycosylation were found. In addition, after Endo H treatment only one peak was detected, with a molecular mass close to the theoretical value of 84 kDa, corroborating the existence of three different glycosylation isoforms of BxTW1 instead of three different isoenzymes. The difference between the molecular mass determined by size exclusion chromatography and SDS-PAGE suggests that BxTW1 works as a non-covalent dimer in its native conformation.

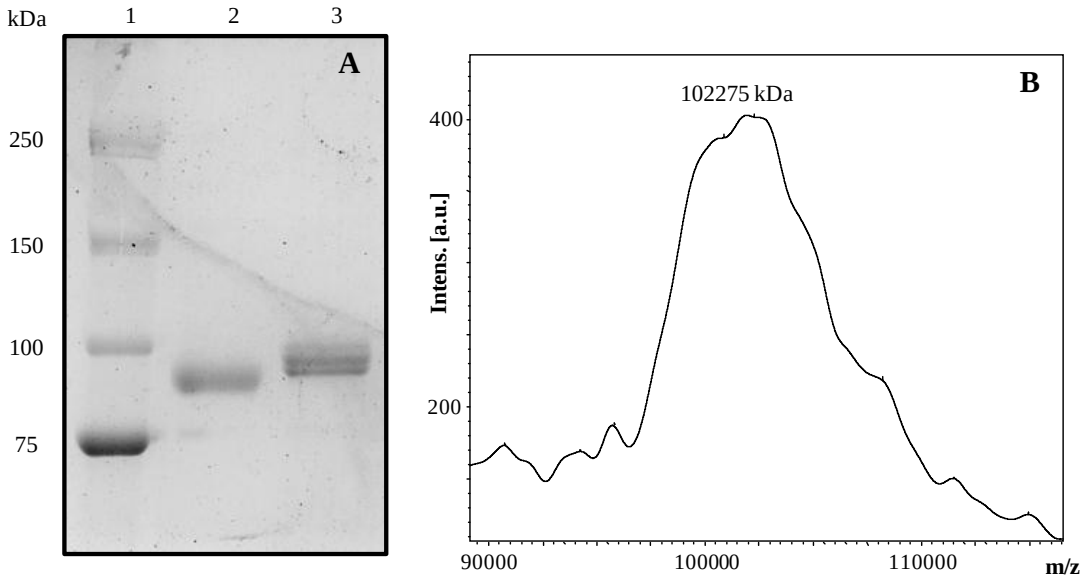


Fig. 4.4. Estimation of BxTW1 molecular mass by SDS-PAGE (A) and MALDI-TOF-MS (B). Lanes 1, molecular mass standards; 2, BxTW1 treated with Endo H; 3, glycosylated BxTW1. Intens., intensity; a.u., arbitrary units.

Isoelectrofocusing indicated that pI of the protein was 7.6, a similar value to those reported for other β-xylosidases (Knob et al., 2010). Nevertheless, the theoretical value obtained from BxTW1 sequence was 4.75. This difference was not surprising since there has been extensively reported that glycosylation can change the isoelectric point of a protein (Marsh et al., 1977).

The influence of temperature and pH on stability and optimal reaction activity of BxTW1 was tested against *p*NPX. The optimum temperature (highest hydrolysis rate) was 70 ºC, although the enzyme lost 70% activity after 30 min at 60 ºC (data not shown). At 50 ºC, the activity loss stabilized around 50% after 1 h (data not shown) and remained stable for 72 h (Fig. 4.5A). The thermal index T50 was 59.9 ºC.

Regarding pH, BxTW1 displayed its maximal activity at pH 3 and exhibited high stability (above 80% of residual activity) between pH 2.2 and 9 for 72 h (Fig. 4.5B).

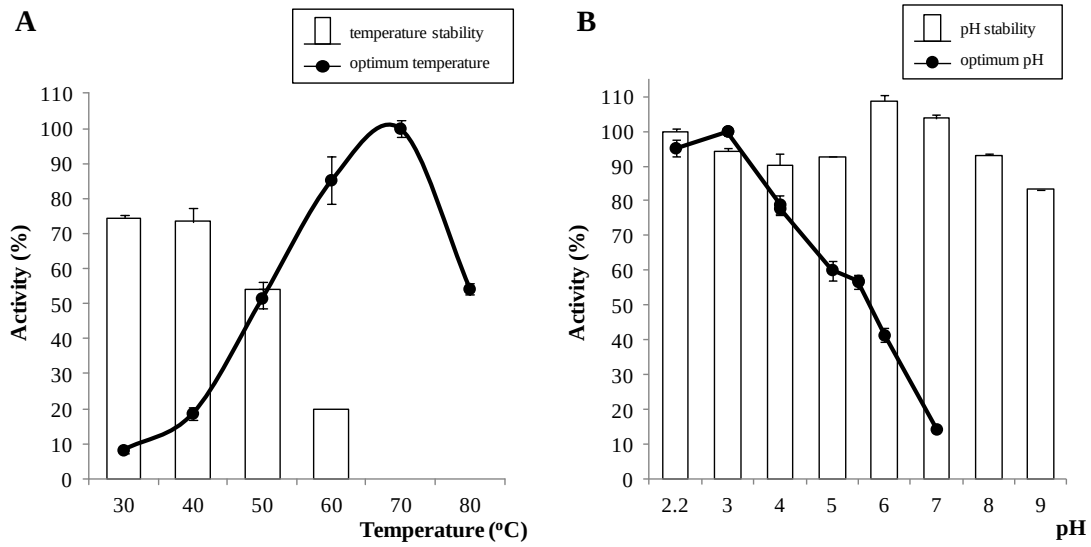


Fig. 4.5. Effect of temperature and pH on BxTW1 activity. A) The line indicates the effect of temperature on enzyme activity and bars show its thermostability in a range from 30 ºC to 70 ºC after 72 h. B) The line corresponds to the effect of pH on enzyme activity and bars show its stability in a range of pH from 2.2 to 9 after 72 h.

Some of these physicochemical properties deserve further explanation. In this sense, the optimum pH value of 3.0 was surprising since most of the described fungal β-xylosidases displayed values from 4.0 to 6.0 (Knob et al., 2010) and few enzymes with this optimum value (Iembo et al., 2002) or lower (Knob and Carmona, 2009) have been described. The causes for this value remain unknown. Sequence alignments of BxTW1 and closely related GH3 xylosidases (data not shown) revealed no changes in the catalytic environment that would explain the low optimum pH of BxTW1. However, Rasmussen *et al.* (2006) reported that β-xylosidases from *T. emersonii* and *T. reesei* changed their optimum pH from 4.0 to 3.0-3.5 when expressed in *Aspergillus oryzae*, for which high *N*-glycosylation potential has been reported (Deshpande et al., 2008). This observation could suggest that this post-translational modification might modulate pH-sensitivity of glycosyl-hydrolases. *N*-oligosaccharides may display charged substituents (Hayes and Varki, 1993) which could affect pH-sensitivity by changing the pI or modifying pKa value of close aminoacids. In the case of BxTW1, *N*-glycosylation has been proven by SDS-PAGE after Endo H treatment and by *in silico* analysis, concluding that the reported difference between theoretical and experimental pI could be explained by these modifications. According to these findings, the low optimum pH of BxTW1 could also be related to its glycosylation pattern and not to changes in the aminoacidic sequence of the active site. The broad stability pattern of the *T. amestolkiae* enzyme was also notable, covering acidic and basic values, while most of the characterized fungal β-xylosidases are quickly inactivated at extreme (low or high) pH values (Poutanen and Puls, 1988; Saha, 2001). Both stability and high activity at

low pH values make it a good candidate to be used in 2G-bioethanol production or as supplements for animal feed.

BxTW1 activity did not show relevant changes in the presence of most of the tested compounds using sodium acetate or citrate buffers at pH 5 (Fig. 4.6). When small inhibition rates were observed, the residual activities were slightly higher in the presence of citrate, probably due to its chelating properties. The most notable results were the slight inhibition registered during the addition of Cu^{2+} and Pb^{2+} in both buffers and the dramatic decrease of activity in the presence of Hg^{2+}. The absence of inhibition in the presence of EDTA, dithiothreitol and 2-mercaptoetanol suggest that BxTW1 does not require metallic cations for its catalytic activity and the absence of a disulfide bond near or inside the active site. The non-dependence of metal cofactors is a common feature of GH3 proteins, but there are a few solved structures displaying disulfide bonds within this group (Varghese et al., 1999). Most importantly, the tolerance to the presence of several heavy metals commonly inactivating β-xylosidases merits especial attention. The resistance is particularly important in the case of Cu^{2+}, which has been reported as a strong inhibitor of many β-xylosidases (Andrade et al., 2004; Saha, 2003) present in the ash content of different lignocellulosic biomasses, showing inhibitory effects on cellulases and reducing the final yield of 2G bioethanol production even at low concentrations (Bin and Hongzhang, 2010).

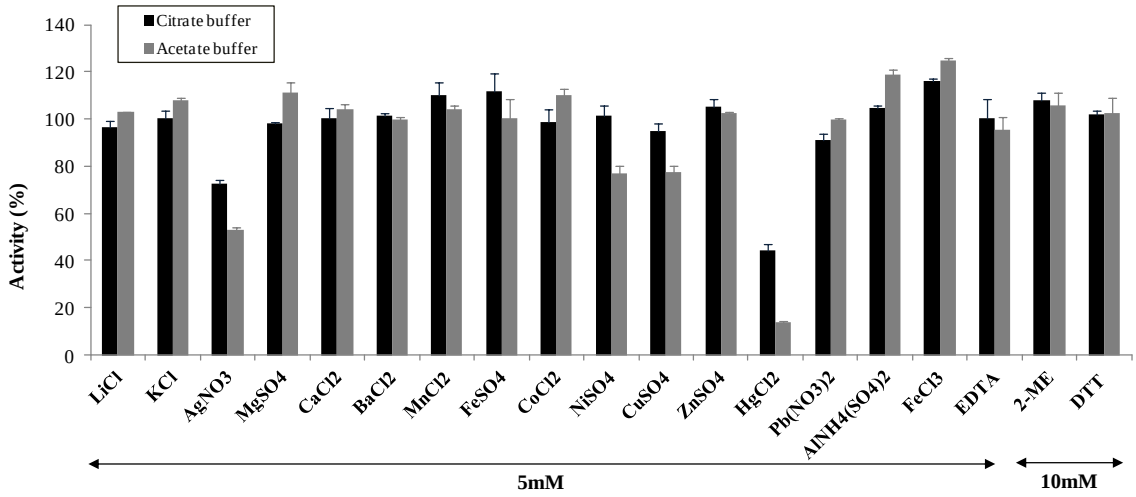


Fig. 4.6. Effect of some chemical compounds on BxTW1 activity.

4.1.5. Substrate specificity and kinetics

The hydrolytic mechanism of BxTW1 was ascertained by ^{1}H-NMR, analyzing xylose release during the first minutes of reaction. The spectra showed a doublet with a chemical shift of 4.51 ppm at 1 min. The new signal was caused by the release of anomeric D-xylopyranose in beta configuration and its intensity increased until 60 min. Then, a new doublet, corresponding to the alpha anomer, was detected. It showed a chemical

shift of 4.77 ppm and was due to mutarotation between alpha and beta anomers of xylopyranose. These results proved that, as all the GH3 family members, BxTW1 worked with a retaining mechanism.

The enzyme hydrolyzed *p*NPX, *p*NP-Arap, *p*NP-Araf, xylooligosaccharides from X2 to X6, and was capable of releasing xylose from beechwood xylan. Nevertheless, no activity was detected on other nitrophenyl substrates or disaccharides assayed. The kinetic parameters of BxTW1 (Table 4.1) were determined using the specific substrates reported above. Although the enzyme was able to hydrolyze *p*NP-α-L-arabinoside independently of the glycon moiety configuration, its affinity towards these substrates was much lower than that found for *p*NPX.

Table 4.1. Kinetic parameters of BxTW1 against different substrates.

Substrate	K_m **(mM)**	V_{max} **(U/mg)**	k_{cat} **(s^{-1})**	k_{cat}/K_m **($mM^{-1}\cdot s^{-1}$)**
*p*NPX	0.17±0.01	52.0±0.5	173	1000
*p*NP-Arap	3.6±0.3	66.9±4.2	220	62
*p*NP-Araf	5.8±0.4	43.0±1.7	143	25
Xylobiose	0.48±0.05	55.2±1.3	183	380
Xylotriose	0.22±0.01	19.8±0.3	66.1	290
Xylotetraose	0.20±0.01	15.4±0.1	51.2	260
Xylopentaose	0.20±0.01	11.8±0.2	39.2	200
Xylohexaose	0.22±0.01	9.5±0.1	32	140
Xylan	7.0±0.2[a]	68.7±0.6	229	-

[a] The K_m for xylan is expressed in milligrams per milliliter.

Even though the maximum velocity of BxTW1 was comparable to those reported for other fungal β-xylosidases (Table 4.2), the results showed a remarkable high affinity of the enzyme to *p*NPX. Very few characterized β-xylosidases, as BXTE from *T. emersonii* (Eisenthal et al., 2007), had a slightly lower K_m value towards this substrate. Nevertheless, BxTW1 demonstrated better kinetic properties: its V_{max} is 22-fold higher compared with Xyl I and the k_{cat} against *p*NPX was 173 s^{-1}, more than 700-fold higher than the reported for BXTE. Catalytic efficiency, an extensively used parameter for enzyme comparison (Eisenthal et al., 2007) is also shown for each enzyme, when available, in Table 4.2. The efficiency of BxTW1 showed to be among the highest values reported. In fact, β-xylosidase from *Bacillus pumilus*, commercialized by Megazyme (SKU code E-BXSEBP), shows a catalytic efficiency of 230 $mM^{-1}\cdot s^{-1}$, calculated from the reported data (Mozolowski and Connerton, 2009), a value 4.5-fold lower than that of BxTW1.

Table 4.2. Comparative kinetic parameters for different fungal β-xylosidases using *p*NPX as model substrate.

Organism	Enzyme	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)	Reference
Talaromyces amestolkiae	BxTW1	0.17	52.0	173.4	1046.8	This work
Aspergillus awamori	X-100	0.25	-	17.5	70	(Mozolowski and Connerton, 2009)
Aspergillus carbonarius		0.198	3.64	-	-	(Kiss and Kiss, 2000)
Aspergillus japonicus		0.31	114	215.1[a]	693[a]	(Wakiyama et al., 2008)
Aspergillus nidulans		1.1	25.6	76.8[a]	69.8[a]	(Kumar and Ramon, 1996)
Aspergillus ochraceus		0.66	39	-	-	(Michelin et al., 2012)
Aureobasidium spp.	Bxyl	2	940	5500	2750	(Hayashi et al., 2001)
Fusarium proliferatum		0.77	75	-	-	(Saha, 2003)
Fusarium verticilloides		0.85	-	-	-	(Saha, 2001)
Humicola grisea	Bxyl	0.48	-	-	-	(Mozolowski and Connerton, 2009)
	Bxyl	1.37	12.98	$1.217\cdot10^{-5}$	0.00001	
Humicola insolens		1.74	22.17		3901	(Mozolowski and Connerton, 2009)
Penicillium sclerotium		0.78	0.51	1.22[a]	1.56[a]	(Knob and Carmona, 2011)
Scytalidium thermophilum	Bxyl	1.7	88	66[a]	38.8[a]	(Zanoelo et al., 2004)
Sporotrichum thermophile		1.1	114	89.3[a]	-	(Katapodis et al., 2006)
Talaromyces emersonii	BXTE	0.06	-	0.017	0.28[a]	(Rasmussen et al., 2006)
	Xyl I	0.13	1.7	426	3276.9	(Mozolowski and Connerton, 2009)
	Xyl II	32.9	6.3	898	27.3	
	Xyl III	1.4	0.26	61	43.6	
Talaromyces thermophilus	Bxyl	2.37	0.049	0.037[a]	0.016[a]	(Guerfali et al., 2008)
Trichoderma reesei	BXTR	0.8	-	0.0147	0.02	(Mozolowski and Connerton, 2009)
Trichoderma viride		5.8	-	21.3	3.7	(Matsuo and Yasui, 1984)

[a] Not included in the original article but calculated with the data provided.

Regarding natural substrates, the enzyme hydrolyzed XOS of different chain length, with similar affinity from 3 to 6 xylose units, but with decreasing catalytic efficiency. The activity of this glycosidase towards xylobiose is in the range of the highest values found in literature (Jordan and Wagschal, 2010), although it showed higher affinity towards longer substrates (X3-X6). Although kinetic characterization of β-xylosidases against XOS with a DP higher than xylobiose has not been deeply studied, a detailed comparison revealed that BxTW1 had the highest catalytic efficiency for all the XOS tested from X3 to X6. In fact, the kinetic constants of BxTW1were frequently one or two orders of magnitude over those of characterized β-xylosidases (Table 4.3).

Table 4.3. Comparison of catalytic efficiencies against XOS from X2 to X3 of fungal and bacterial β-xylosidases.

Organism	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)					Reference
	X2	X3	X4	X5	X6	
Talaromyces amestolkiae	382	287	258	198	143	This work
Alkaliphilus metalliredigens	39.4	30.7	-	-	-	(Jordan et al., 2013)
Aspergillus nidulans	70 14	58 9	42 8	33 5	22 4	(Dilokpimol et al., 2011)
Aspergillus oryzae[a]	13.8	9.7	33.1	-	-	(Kirikyali et al., 2014)
Bacillus pumilus *Bacillus subtilis*	7.45 56.6	6.10 35.2	1.42 1.42	- -	- -	(Jordan et al., 2013)
Geobacillus thermoleovorans	$5.1\cdot10^{-3}$	$3.9\cdot10^{-3}$	-	-	-	(Wagschal et al., 2009)
Lactobacillus brevis	138	80.8	2.40	-	-	(Jordan et al., 2013)
Neurospora crassa[a]	3.4	1.4	0.7	-	-	(Kirikyali and Connerton, 2014)
Selenomonas ruminantium	90.2	44.8	33.3	27.0	26.1	(Jordan, 2008)

[a] Recombinant protein expressed *in P. pastoris*

In addition, BxTW1 showed activity against beechwood xylan, something uncommon among most of the known β-xylosidases. These behaviors have been previously reported and they are considered a typical feature of exo-type xylanolytic enzymes (Herrmann et al., 1997; Sunna and Antranikian, 1997), in opposition to classical β-xylosidases (Sunna and Antranikian, 1997). Exo-type xylanases (EC 3.2.1.156) are also called reducing end xylose-releasing exo-oligoxylanases or Rex enzymes and, as described in the Introduction, they share with β-xylosidases (EC 3.2.1.37) the exo-attack of substrates. Nevertheless, there are several differences suggesting that BxTW1 should be identified as a β-xylosidase. As

mentioned above, alignment studies displayed high homology between BxTW1 and other putative and characterized β-xylosidases. Moreover, all the reported Rex enzymes are included in GH8 family, work with inversion of the configuration and are unable to hydrolyze xylobiose (Juturu and Wu, 2014). These data strongly suggest that BxTW1 cannot be considered as a Rex enzyme, and should be considered a β-xylosidase.

One of the most remarkable data displayed above was that the enzyme attacked X3-X6 with higher affinity than X2. Since K_m values were calculated by estimating released xylose instead of monitoring substrate consumption, and in order to confirm that BxTW1 hydrolyzed X3 preferentially over X2, xylotriose consumption and xylobiose generation were followed by ^{1}H-NMR spectroscopy (Fig. 4.7A). Comparison of spectra revealed the preference of BxTW1 for the trisaccharide over the released disaccharide (Fig. 4.7B). This result unequivocally demonstrated xylotriose consumption and agreed with global K_m values for XOS calculated from the xylose released. Enzyme inhibition by product was also studied, revealing that the activity against *p*NPX was competitively inhibited by xylose, with a K_i of 1.7 mM.

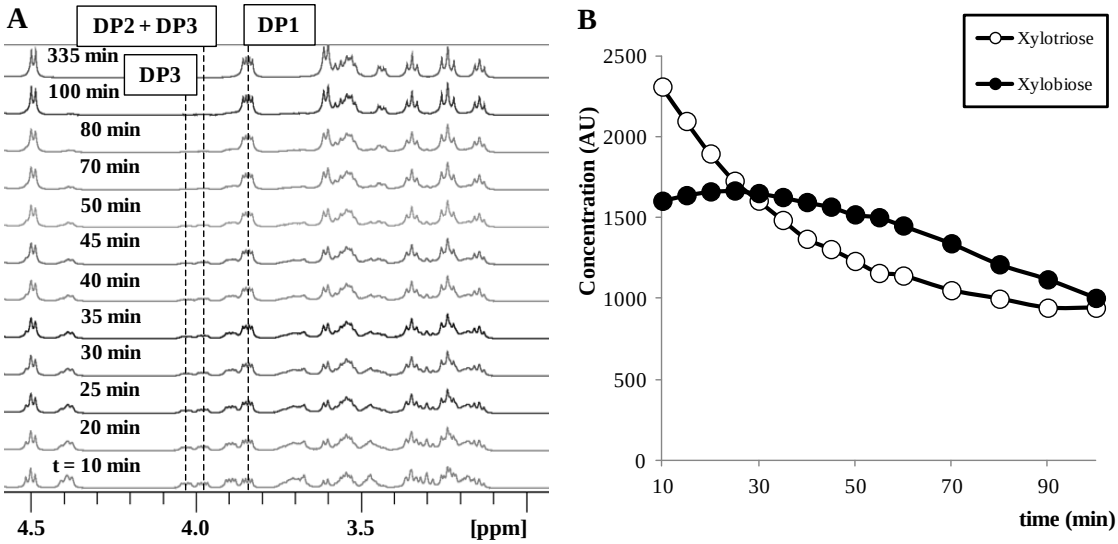


Fig. 4.7. A) Proton NMR spectra of xylotriose consumption by BxTW1 along time. Signals using for quantification are pointed. B) Evolution of xylotriose and xylobiose concentration during the reaction time. Concentrations were determined by integrating the appropriated signals of each compound.

4.1.6. Transxylosylation

The transxylosylation capabilities of BxTW1 were tested. Xylotriose or xylobiose were firstly assayed as simultaneous donors and acceptors in separated reactions. This double role of substrates has been previously reported (Kurakake et al., 2005). Since the enzyme preferentially hydrolyzes X3 over X2 (Fig. 4.7), differences in transxylosylation rates

were also analyzed as a function of the acceptor length and concentration. In this work, a direct relation between acceptor concentration and the synthesis of transxylosylation products was observed (Fig. 4.8). On the other hand, xylotriose was synthesized from xylobiose and, when xylotriose was used as the substrate, the resultant product was xylotetraose. In both cases transxylosylation ratios increased with the substrate concentrations (detection limit above 5 mM substrate). Below 10 mM, transxylosylation rates were comparable using X2 or X3. However, X2 was better transxylosylation acceptor than X3 at concentrations over 20 mM (about 40% transxylosylation rate versus 30%, respectively). Figure 4.8 shows the evolution of transxylosylation and hydrolysis ratios using xylobiose (Fig. 4.8A) and xylotriose (Fig. 4.8B) as substrates.

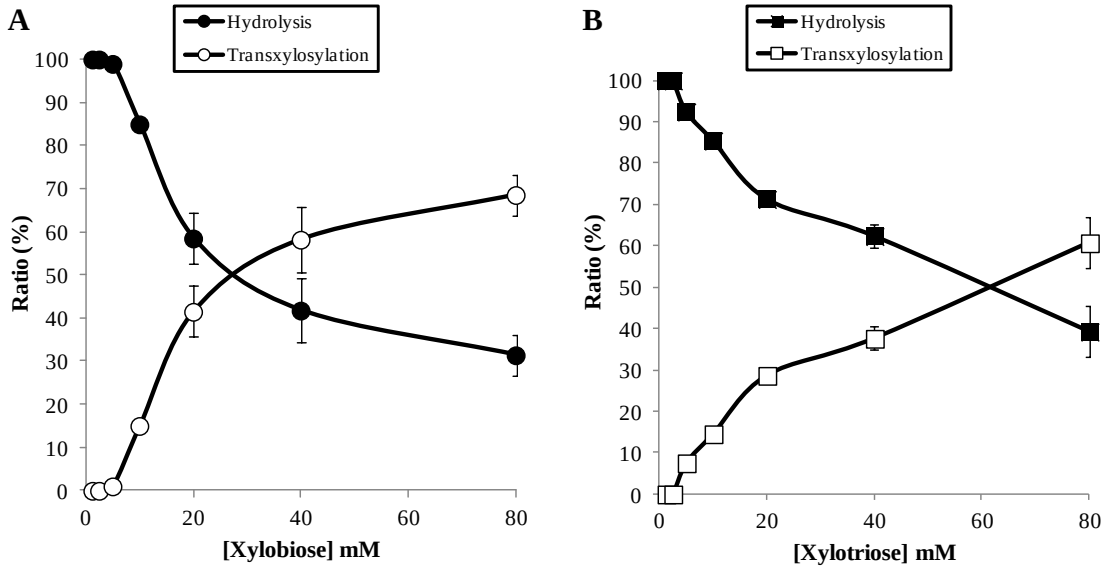


Fig. 4.8. Transxylosylation ratios according to the initial substrate concentration. Reaction products and substrate were separated by HPLC. Ratios were obtained from comparing areas under the curves of remaining substrate and product of transxylosylation.

Attending to these data, it can be concluded that BxTW1 demonstrated transxylosylation capacity, and rates increased with substrate concentration when xylobiose and xylotriose were used as donor and acceptor simultaneously. The transxylosylation and hydrolytic rates were complementary, since the longest substrate was a worse acceptor than the shortest.

The transxylosylation specificity of BxTW1 was tested in reactions with *p*NPX as donor and a large excess of different acceptors, measuring xylose/*p*NP ratios at the final reaction time. To calculate transxylosylation rates, the stoichiometric relation between products (xylose and *p*NP) was taken as 1:1. Then, detection of *p*NP in a significantly higher concentration than xylose for an assayed acceptor indicates that transxylosylation

occurred and the monosaccharide has been attached to the acceptor. A variety of alkyl alcohols, sugar alcohols, monosaccharides and disaccharides were tested as acceptors. A small transxylosylation rate of 13% was observed in the absence of acceptor, showing that BxTW1 was capable of using *p*NPX molecules as acceptors. The consumed substrate exceeded 80% in all cases, and the highest transxylosylation rates were obtained mainly with alkan-1-ols, alkan-2-ols, and sugar alcohols (Fig. 4.9), while monosaccharides and disaccharides turned out to be the worst acceptors. Chemical similarities between acceptors were estimated by Tanimoto coefficient calculation and a comparative analysis was carried out using the hierarchical clustering tool from the MatLab environment (Fig. 4.10).

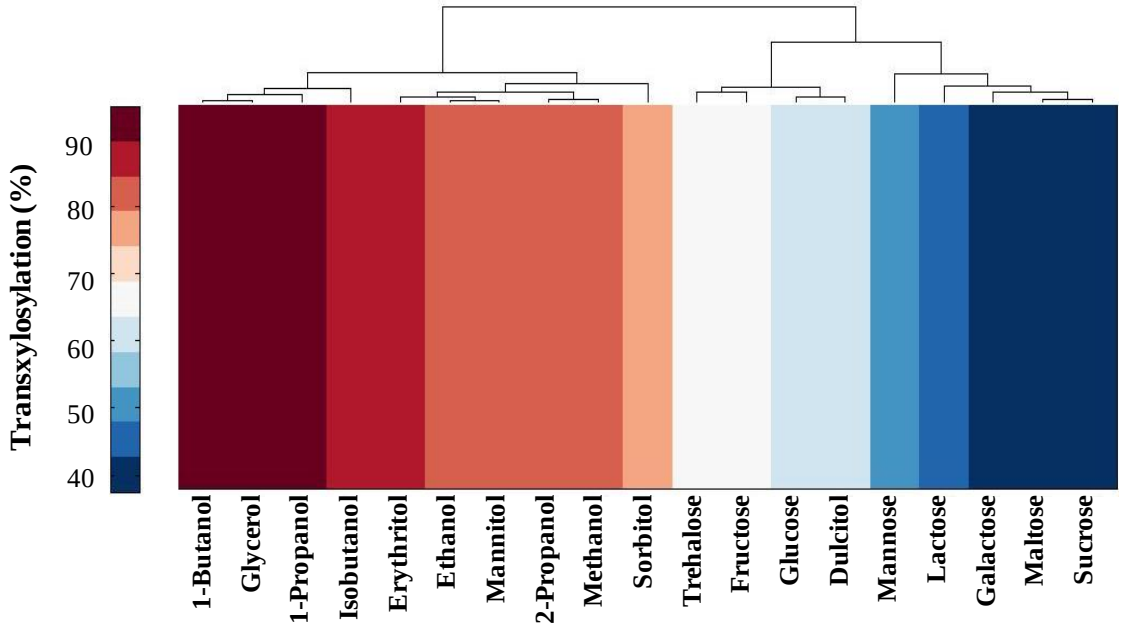


Fig. 4.9. Transxylosylation ratios of BxTW1 in the presence of different acceptors. Acceptor specificity is presented as a heat map based on transxylosylation ratios. The hierarchical clustering analysis was performed using the clustergram algorithm within Matlab environment (MathWorks, Natick, MA).

The results showed that compounds with very close physicochemical features behave differently as transxylosylation acceptors. Regarding sugar alcohols, mannitol is a much better acceptor than sorbitol and dulcitol although all of them have the same molecular formula. In the case of aldoses, glucose, galactose and mannose also share the same empirical formula, but were very different as acceptors: glucose was the most efficient, while transxylosylation yields for galactose were significant lower.

Regioselectivity of BxTW1 when catalyzing the formation of a new glycosidic linkage, using xylobiose or xylose as acceptor, was also investigated. Xylobiose was used as simultaneous donor and acceptor for

the synthesis of either the trisaccharide or higher DP transxylosylation products. A DOSY-NMR spectrum of the reaction mixture was acquired, and the detected signals could be correlated with the presence of mono-, di- and trisaccharides. DOSY-TOCSY and ^{1}H-^{13}C HSQC-NMR spectra were acquired in order to simplify the assignation of ^{1}H 1D-NMR signals. The chemical-shift displacement data allowed concluding that BxTW1 catalyzed the regioselective synthesis of 1,4-β-D-xylotriose as unique transxylosylation product. BxTW1 regioselectivity was also tested using *p*NPX as donor and xylose as acceptor, to test if the reaction products were disaccharides or had higher DPs. A ^{1}H-NMR spectrum was acquired from the reaction mix and ^{1}H-^{13}C HSQC-NMR data were used to simplify the analysis. The assignation of signals indicated that BxTW1 catalyzed the synthesis of 1,4-β-D-xylobiose, as the unique transxylosylation product.

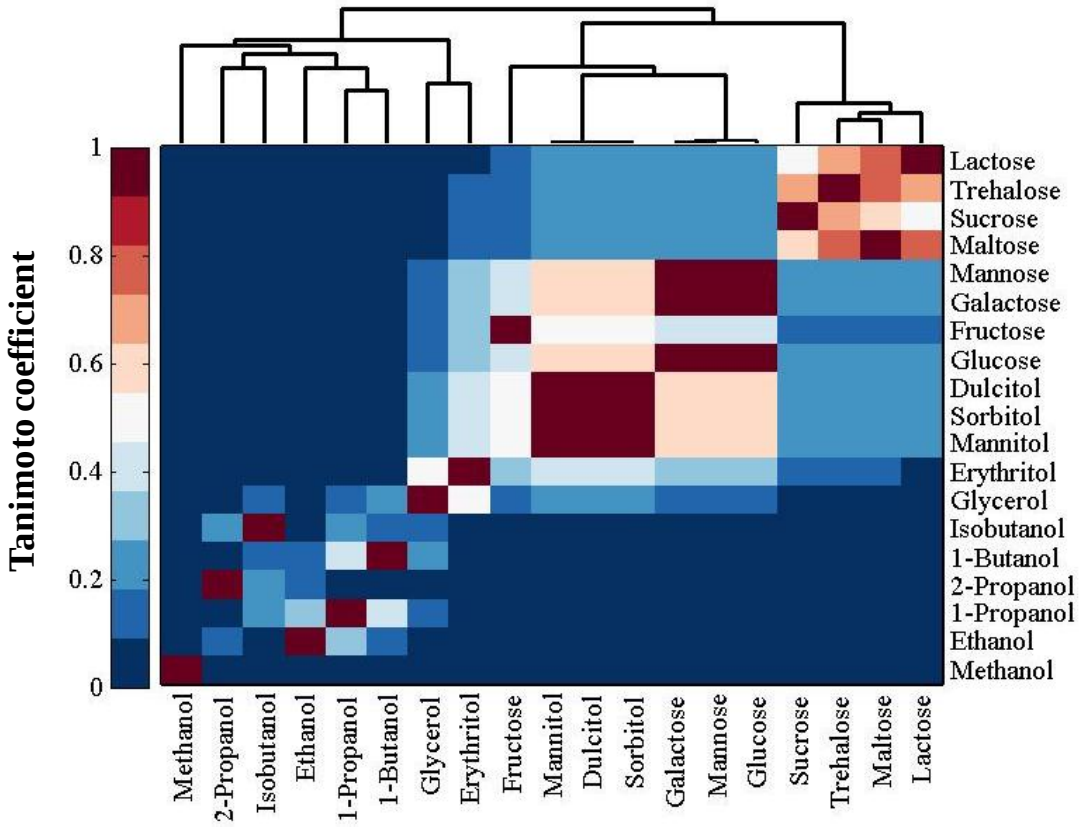


Fig. 4.10. Hierarchical clustering of acceptors' chemical similarity estimated by Tanimoto coefficient calculation using Chemmine program. Clustering was performed within Matlab environment (MathWorks, Natick, MA).

Therefore, BxTW1 showed broad acceptor specificity, high efficiency and, what is even most importantly, remarkable regioselectivity. Short alkan-ols were the best acceptors, probably due to their low molecular mass and to the physicochemical properties of the enzyme's active site, as its size or hydrophobicity. The results suggested that aldoses and alcohols were preferentially transxylosylated on primary alcohols,

since 1-propanol and 1-butanol were better acceptors than 2-propanol and isobutanol, respectively. To confirm this, aldohexoses distinguished only by their three-dimensional spatial orientation were used as acceptors. In D-glucose all hydroxyl groups but the primary one (C6) are in equatorial position and, hence, the transxylosylation rates were higher than those obtained with D-mannose, where C2 hydroxyl group shares the axial position with the primary alcohol. The transxylosylation rate was even lower when D-galactose was used as acceptor, where the axial position was occupied by C4 hydroxyl (closer than C2 to the primary alcohol). No clear conclusions could be drawn from the results obtained when sugar alcohols or disaccharides were used as acceptors; in these cases, unknown steric hindrances may occur. A deeper understanding of the residues and mechanism involved in transxylosylation reactions would be necessary to decipher acceptor specificity (Dilokpimol et al., 2011; Kurakake et al., 2005)

Both, promiscuity and efficiency, suggest a considerable potential of BxTW1 for the biosynthesis of oligosaccharides with pharmacological or industrial interest. The enzymatic synthesis of new oligosaccharides by transglycosylation is a promising alternative to chemical methods. Many glycosidases have been studied in order to determine their ability to form a glycosidic bond stereospecifically, but most of them show a low regioselectivity. This implies that the transglycosylation products are multiple instead of unique, hence hampering their use for industrial production. Few regioselective glycosidases have been described and it has been related with their specificity (Mala et al., 1999). In this sense, although BxTW1 regioselectivity has been analyzed only when xylose or xylobiose were used as acceptors, its broad substrate specificity makes it a good candidate to test different and new molecules as final xylose receivers. This reinforces the potential of BxTW1 for the biosynthesis of new oligosaccharides with potential industrial interest.

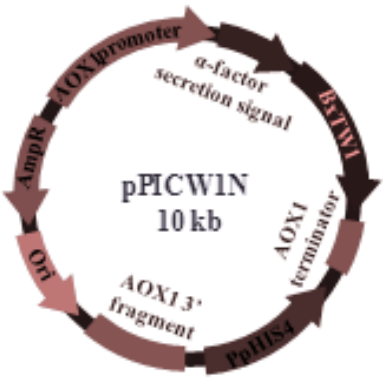
4.1.7. Sequencing, classification and molecular characterization of BxTW1

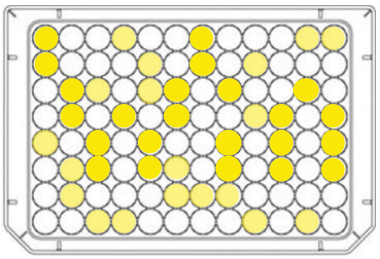
The preliminary identification of BxTW1 was based on its peptide mass fingerprint. The three bands identified in SDS-PAGE gels as glycosylated isoforms of BxTW1were analyzed, giving the same profile of tryptic peptides. The homology search of these peptides revealed the closeness of BxTW1 with four putative fungal β-xylosidases from *T. stipitatus* ATCC 10500 (gi:242771939), *T. cellulolyticus* (gi:348604625) *Talaromyces marneffei* ATCC 18224 (gi:212531051) and *Hypocrea orientalis* strain EU7-22 (gi:380293099), and three β-xylosidases isolated from *Trichoderma reesei* (gi:2791277), *Talaromyces emersonii* (gi:48526507) and *Aspergillus fumigatus* (gi:76160897), respectively.

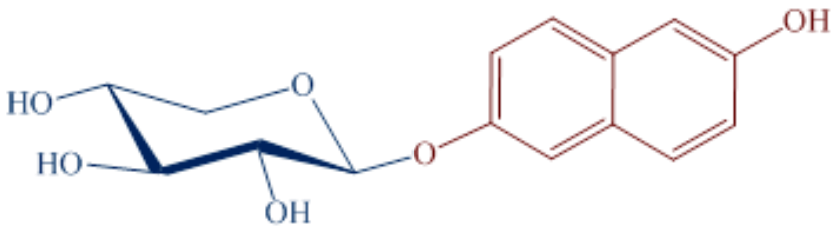
Gene sequencing revealed that a 2,394 bp region with no introns codifies for BxTW1. The nucleotide sequence was submitted to the GenBank database with accession number KP119719. An homology search based on DNA sequence showed its high identity with putative β-xylosidases from *T. stipitatus* ATCC 10500 (gi:242771939), *T. cellulolyticus* (gi:348604625) and *T. marneffei* ATCC 18224 (gi:212531051), all of them belonging to the GH3 family and lacking introns. The gene sequence was then submitted to the dbCAN server in order to annotate the enzyme into a glycosyl hydrolase family, confirming that BxTW1 belongs to GH3.

4.2. CHAPTER II

ENZYMATIC FINE-TUNING FOR 2-(6-HYDROXYNAPHTHYL) β-D-XYLOPYRANOSIDE SYNTHESIS CEATALYZED BY THE RECOMBINANT β-XYLOSIDASE BXTW1 FROM *Talaromyces amestolkiae*







4.2.1. Background

The efficient synthesis of glycosides remains a significant challenge, especially for reactions that must be done on a large scale. Glycosides play important roles in biology. For example, the glycan moiety of glycoproteins and glycolipids is essential for many physiological processes, such as immune responses, cell adhesion or protein folding (Mattner et al., 2006; Shental-Bechor and Levy, 2009; Wang and Huang, 2009). In addition, non-natural glycosides have been synthesized for a wide variety of applications, for example to enhance the properties of antioxidants or to generate new antibiotics (Baltz, 2006; Regev-Shoshani et al., 2003).

In the case of β-xylosidases and transxylosylation the use of xylobiose or other xylooligosaccharides, derived from the hydrolysis of xylan, may be a cost-effective sugar donor (Jain et al., 2014). The attachment of a xylose moiety to a specific acceptor can result, for instance, in novel surfactants (Matsumura et al., 1998), antithrombotic drugs (Toomey et al., 2006) or primers for studying the biosynthesis of heparan sulfate (Lugemwa and Esko, 1991). In fact, this priming role in the formation of glycosaminoglycans led to the development of specific xylosides as anti-proliferative compounds (Kalita et al., 2015; Nilsson et al., 2009), which have been successfully tested as selective inhibitors of the growing of tumor cells both *in vitro* and *in vivo* assays, as is the case for 2-(6-hydroxynaphthyl) β-D-xylopyranoside (Mani et al., 2004).

The purification and characterization of the BxTW1 β-xylosidase from *T. amestolkiae* has been reported in the former chapter. The enzyme displays remarkable affinity and activity against xylobiose and XOS but, most importantly, it shows broad acceptor versatility and high regioselectivity in catalyzing transxylosylation reactions. However, the amount of protein secreted by the native producer was quite low, which, when coupled with the poor purification yields reported severely limited its potential application. This chapter reports the expression of high levels of the recombinant β-xylosidase (rBxTW1) in the methylotrophic yeast *P. pastoris*. In addition, the work describes the rBxTW1-catalyzed synthesis of 2-(6-hydroxynaphthyl) β-D-xylopyranoside using xylobiose as xylosyl donor and 2,6-dihydroxynaphthalene (2,6-DHN) as acceptor. To the best of our knowledge, this is the first report on enzymatic synthesis of this xyloside, which is known as a selective anti-proliferative compound (Mani et al., 2004). The optimization of the reaction parameters by a Box-Behnken design (BBD) is also presented (Box and Behnken, 1960).

4.2.2. Expression of rBxTW1 in *P. pastoris*

The gene *bxtw1* was successfully expressed in *P. pastoris*. The mature *bxtw1* sequence without signal peptide and introns comprised 2,337 bp, including the native stop codon. After the screening, clone 18 from strain GS115 proved to be the best rBxTW1 producer and was selected for enzyme production in liquid cultures.

β-Xylosidase activity reached a maximum of 8 U/mL in 10-day-old YEPS cultures (Fig. 4.11), which is an excellent value when compared with those reported for other fungal β-xylosidases of the GH3 family produced in *P. pastoris* (Table 4.4).

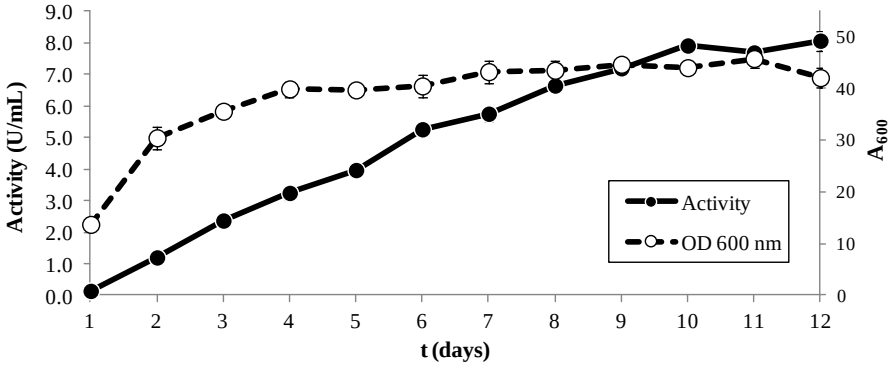


Fig. 4.11. Extracellular β-xylosidase activity and absorbance at 600 nm of *P. pastoris* cultures in YEPS medium with 5 g/L methanol.

Table 4.4. Comparative production data of GH3 fungal β-xylosidases heterologously expressed in *P. pastoris*.

Enzyme	Source	Production (U/mL)	Reference
rBxTW1	*Talaromyces amestolkiae*	8.0	This work
AnBX (Opt)	*Aspergillus niger*	4.62	(Choengpanya et al., 2015)
Xyl3A	*Humicola insolens*	1.16[a]	(Xia et al., 2015)
AnXln3D	*Aspergillus niger*	6.46	(Li et al., 2015a)
NCU09923	*Neurospora crassa*	3.04[a]	(Kirikyali and Connerton, 2014)
AN2359.2	*Aspergillus nidulans*	3	(Vasu et al., 2012)
Bxl1	*Aureobasidium pullulans*	14.9	(Ohta et al., 2010)
XylA	*Aspergillus japonicus*	0.33	(Wakiyama et al., 2008)

[a] Not included in the original article but calculated with data provided.

The recombinant rBxTW1 was completely purified by FPLC after a single step of cation-exchange chromatography with a yield of 91.5% and a degree of purification of 1.9. The above data represent a huge increase in both maximal activity and protein purification yield respect to the native enzyme, which is produced at 0.8 U/mL and purified after three chromatographic steps, with a final yield of 10.8% (Chapter I). These results indicate that *P. pastoris* is an appropriate host for producing rBxTW1.

4.2.3. Characterization of rBxTW1

The isoelectric point of the recombinant enzyme was determined to be 8.4, more basic than that of the native enzyme, which is probably due to their different glycosylation pattern (Marsh et al., 1977). The molecular mass estimated by SDS-PAGE was around 100 kDa. The difference between this value and that predicted from the amino acid sequence of the protein (84.650 kDa) suggested that rBxTW1 was glycosylated, which is common for proteins expressed in *P. pastoris* (Macauley-Patrick et al., 2005). The enzyme was *N*-deglycosylated with Endo H and subsequently analyzed by SDS-PAGE and MALDI-TOF MS (Fig. 4.12).

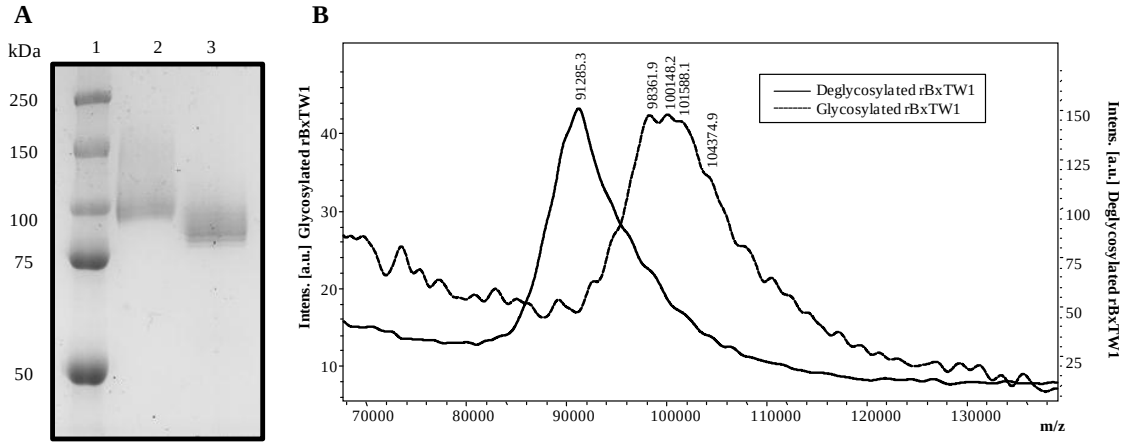


Fig. 4.12. Estimation of rBxTW1 molecular mass by (A) SDS-PAGE and (B) MALDI-TOF MS. Lanes: 1, molecular mass standards; 2, glycosylated BxTW1; 3, BxTW1 treated with Endo H. Intens., intensity; a.u., arbitrary units.

As expected, the molecular mass decreased, displaying a value of 91 kDa by MALDI-TOF. The difference with respect to the theoretical 84.650 kDa may be attributed to *O*-glycosylation which are also introduced by the yeast but not removed with Endo H. These assays confirmed that *P. pastoris* was producing the enzyme as a glycoprotein with approximately 10% *N*-glycosylation. On the other hand, the measured molecular mass of rBxTW1 determined by size exclusion chromatography was around 160 kDa, suggesting that in aqueous medium this protein forms a non-covalent dimer, as reported for the native enzyme (Chapter I). As the values obtained by size exclusion chromatography are influenced by the shape of the proteins, and are not fully accurate, hereinafter the correct average mass of the glycosylated monomeric rBxTW1 will be assumed to be 100 kDa, as determined by SDS-PAGE, and that of the dimeric form 200 kDa. This molecular mass value is the one used to calculate the kinetic parameters of the β-xylosidase.

The kinetic properties of rBxTW1 were determined against *p*NP-pentoses, XOS and beechwood xylan (Table 4.5). The enzyme showed high affinity for *p*NPX and XOS, especially when compared with the increased

K_m displayed in the hydrolysis of *p*NP-Arap and *p*NP-Araf. Competitive inhibition by product (xylose) was analyzed resulting in a K_i of 1.7±0.3 mM when *p*NPX was used as substrate.

Table 4.5. Kinetic parameters of rBxTW1.

Substrate	K_m (mM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}·s^{-1}$)
*p*NPX	0.20±0.01	20.8±0.3	69.3	336
*p*NP-Arap	1.6±0.1	8.3±0.2	28	17
*p*NP-Araf	4.3±0.3	14.9±0.4	49.8	12
Xylobiose	0.51±0.03	26.4±0.3	87.9	170
Xylotriose	0.19±0.01	9.0±0.1	30	160
Xylotetraose	0.20±0.01	6.34±0.06	21.1	100
Xylopentaose	0.22±0.01	4.48±0.05	14.9	68
Xylohexaose	0.21±0.02	4.27±0.07	14.2	69
Xylan	8.9±0.3[a]	21.4±0.3	71.5	-

[a] The K_m for xylan is expressed in milligrams per milliliter.

The biochemical and kinetic properties of rBxTW1, namely the general profiles for optimal pH (Fig. 4.13A) and temperature (Fig. 4.13B), substrate specificity, and high affinity for *p*NPX and XOS, were very similar to those reported for the native enzyme (Chapter I). However, the temperature for maximal activity decreased from 70 °C to 60 °C, T50 was around 9 degrees lower and the enzyme lost its stability at basic pH, indicating the lower stability of the recombinant form. In addition, a decrease of its maximum velocity was also observed. Similar results have been reported by Wei *et al*. expressing a fungal GH3 β-glucosidase in *P. pastoris* (2013), which were attributed to the fact that *N*-glycans usually introduced by this yeast are larger than those of fungi.

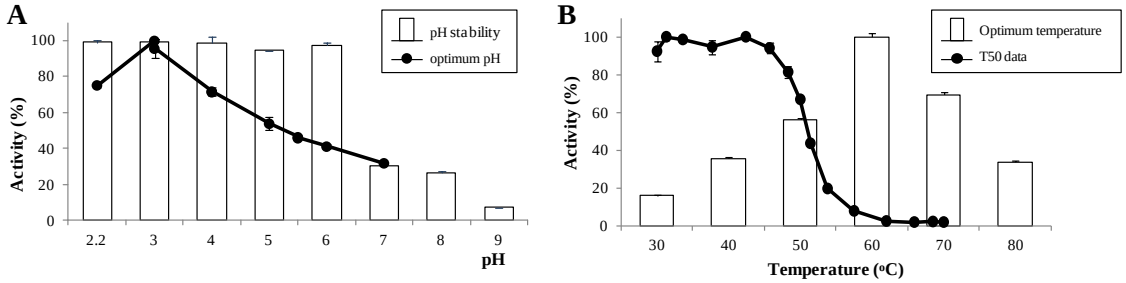


Fig. 4.13. Effect on rBxTW1 activity of: (A) pH and (B) temperature. (A) The line indicates the effect of pH on enzyme activity, and the bars show its stability over a range of pH values from 2.2 to 9 after 72 h. (B) The line displays the evolution of residual activity for T50 determination, and the bars correspond to the effect of the reaction temperature on enzyme activity.

The native and recombinant BxTW1 enzymes both carry glycosylation at approx. 10% of their mass (Chapter I). However, the compositions of their glycans are different (Table 4.6).

Table 4.6. Monosaccharide distribution and linkage types present in the carbohydrate moiety of the native and recombinant BxTW1.

		Content (%)	
Monosaccharide	**Characteristic ions (m/z)**	**BxTW1**	**rBxTW1**
Mannose	43, 115, 145, 187, 217, 259, 361	68.7	91.9
Glucose	43, 115, 145, 187, 217, 259, 361	13.1	1.9
Glucosamine	43, 84, 102, 144, 156, 258, 318, 360	12.1	6.2
Galactose	43, 115, 145, 187, 217, 259, 361	6.1	-
Deduced linkage	**Characteristic ions (m/z)**	**BxTW1**	**rBxTW1**
Man*p*-(1→	87, 88, 102, 118, 129, 161, 205	27.5	29.0
Gal*p*-(1→	89, 101, 102, 118, 162, 205	3.0	0.4
→2)-Man*p*-(1→	87, 88, 101, 129, 130, 161, 190	18.1	35.3
→3)-Man*p*-(1→	101, 118, 129, 161, 234	0.0	2.0
→6)-Man*p*-(1→	87, 88, 99, 102, 118, 129, 162, 189	16.4	3.4
→2,3)-Man*p*-(1→	101, 129, 161, 202, 262	0.0	1.3
→2,6)-Man*p*-(1→	117, 118, 129, 130, 189, 190	1.9	14.0
→3,6)-Man*p*-(1→	118, 129, 189, 174, 234	14.5	8.1
→3,4,6)-Man*p*-(1→	118, 139	6.3	0.5
→4)-Glc*p*NH_2-(1→	117, 159, 233	12.3	4.6

Both enzymes had mannose as the major monosaccharide, but the carbohydrate chains of the β-xylosidase secreted by *T. amestolkiae* also contained substantial amounts of glucose and *N*-acetyl-glucosamine, while in rBxTW1 mannose residues represented more than 90% of its total sugar content, which is consistent with the hyper-mannosylation described for yeasts (Imperiali and O'Connor, 1999). In addition, these enzymes also differed in the linkage types of their glycan component (Table 4.6). The interpretation of the data will be focused on the mannose content, which represents the main part of the glycan and has been deeply studied in the *N*-glycosylation patterns reported for fungi and yeasts (Deshpande et al., 2008; Herscovics, 1999b). Mannose residues with a single link assigned (Man*p*-(1→) represent the non-reducing ends of oligosaccharide chains, two links meant a residue for chain extension (→2)-Man*p*-(1→; →3)-Man*p*-(1→) while three or four links implied a branching point. Based on this, there are important differences between the recombinant and the native fungal enzyme. rBxTW1 displays about 35% of →2)-Man*p*-(1→ units and low content of the other types of extension-residues, while in the native enzyme the proportion of →2)-Man*p*-(1→ and →6)-Man*p*-(1→ units is similar (18 and 16% respectively). On the other hand, the native fungal enzyme was found to have 6% branching points for triple branching, whereas these residues represent less than 1% in rBxTW1. These data

suggest that the glycan in the enzyme from *T. amestolkiae* has a highly branched structure, but with shorter chains in comparison with the recombinant enzyme, which also displayed more homogeneity.

The differences in carbohydrate content and structure between the two enzymes are in good accordance with the hypothesis proposed by Wei *et al*. (2013), suggesting that the long mannose-chains incorporated by *P. pastoris*, in opposition to the shorter and more heterogeneous fungal glycosylation, may be the primary cause of the differences observed between the two glycosidases.

However, the drawbacks observed for the recombinant enzyme are compensated by its high production levels and purification yields, since expression in *P. pastoris* led to an increase of 85-fold on the recovered activity units per volume of culture.

4.2.4. rBxTW1 as a versatile tool for transxylosylation of bioactive compounds

In order to fully determine the biotechnological potential of the recombinant enzyme, its transxylosylation capacities were analyzed. To test if rBxTW1 kept the transglycosylation potential exhibited by the native enzyme (Chapter I), and in particular to assess its aglycone preference, a rapid screen of aglycone specificity was performed, according to the methodology developed by Blanchard and Withers (2001). The library of tested compounds included confirmed acceptors for the native enzyme, representative carbohydrates, alcohols and aromatic compounds together with some amino acids and other chemicals readily available in the laboratory.

The results from this study are gathered as a heat map at Figure 4.14. The high number of positive hits suggested that the recombinant enzyme retained the broad acceptor versatility reported for BxTW1. Sugar alcohols seemed to be very good acceptors for both enzymes. The presence of gallic acid and *p*-nitrophenyl β-D-glucuronide among the few negative hits suggested that carboxylic acids are not suitable acceptors for being transxylosylated by rBxTW1. Xylobiose and xylose also appeared as apparent negative hits, which is at first sight surprising because both carbohydrates are established acceptors for BxTW1 (Chapter I). This is due to the fact that xylose and xylobiose are, respectively, a competitive inhibitor and a substrate for rBxTW1, thus competed with *p*NPX in the assay, thereby appearing not to be acceptors. The same could be true for *p*-nitrophenyl β-D-glucuronide. This emphasizes the need to carry out a parallel screen with non-inactivated enzyme to check for potential inhibitors of this class, so that they can be investigated separately.

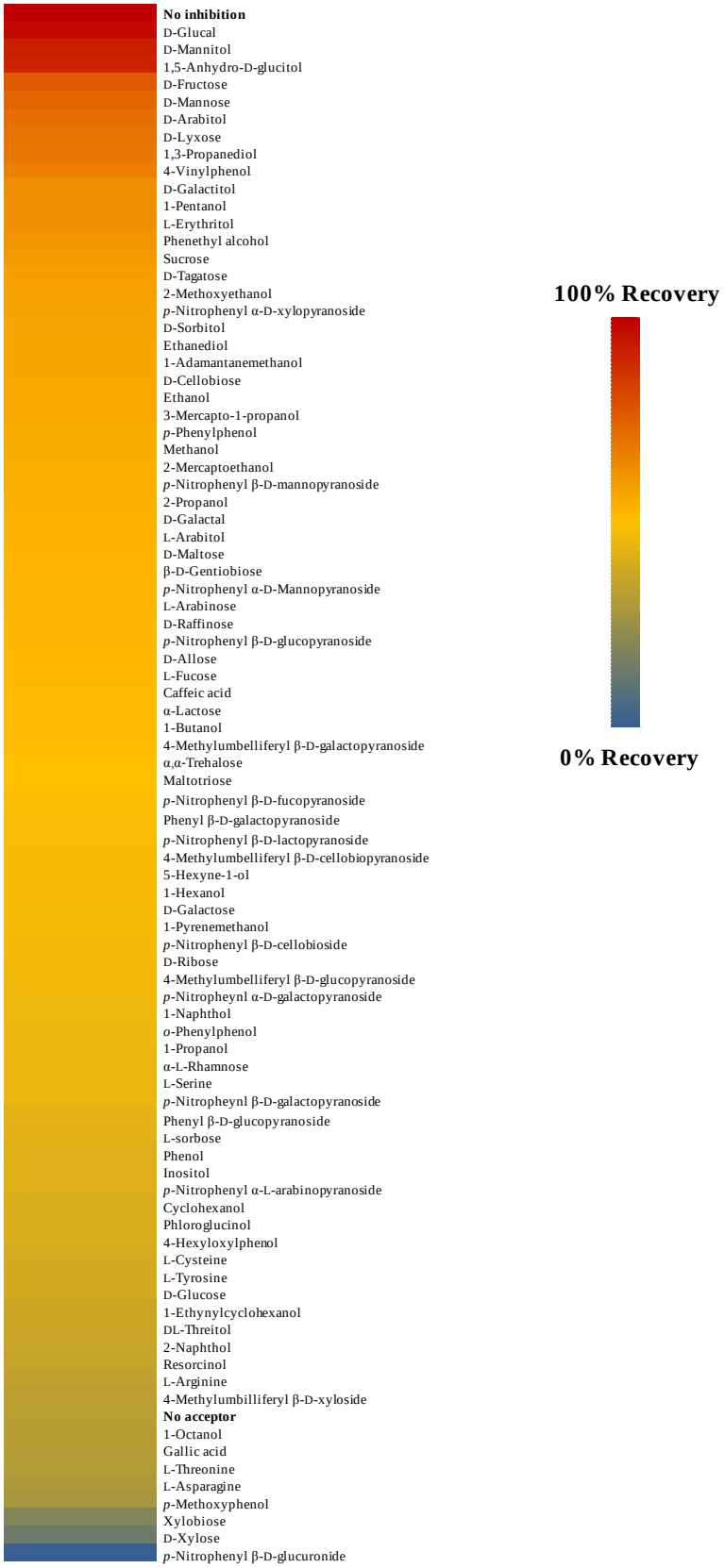


Fig. 4.14. Heat map of inhibition recovery of rBxTW1 in the presence of the assayed compounds. Those compounds giving values between no-acceptor and the no-inhibition controls were considered potential acceptors of transxylosylation.

Among the most clear positive hits there were at least three remarkable compounds for their potential biotechnological importance in terms of transglycosylation. 4-vinylphenol is a volatile phenolic compound found in wine, beer and other non-alcoholic beverages as orange juice. It is released from precursors during storage, being one of the main causes of off-flavor problems. Transxylosylation may provide a solution to thisindustrial concern by converting this molecule into a stable non-volatile glycoside (Fallico et al., 1996; Vanbeneden et al., 2008). The second interesting compound is 1-pentanol that can be transformed into pentyl xyloside, an alkyl xyloside that, like others, may be a useful surfactant (Ochs et al., 2011). Regarding phenethyl alcohol, its antimicrobial properties are well known and natural glycosides of this compound have been found and reported to display immunosuppressive responses (Corre et al., 1990; Saeidnia et al., 2009). The chemical synthesis of glycosides from 4-vinylphenol, 1-pentanol and phenethyl alcohol needs a minimum of three steps. Therefore, the easily accessible synthesis of those xylosides and related derivatives by transxylosylation and the study of their potential biological activity may lead to a better understanding of their properties. To the best of our knowledge, there is only one precedent of using phenethyl alcohol (Shinoyama et al., 1988) and no previous report concerning the use of 4-vinylphenol as acceptor for enzymatic transglycosylation.

4.2.5. Enzymatic synthesis of 2-(6-hydroxynaphthyl) β-D-xylopyranoside and production enhancement by RSM

The screening of the aglycone specificity also revealed 2-naphthol and especially 1-naphthol as potential transxylosylation acceptors for rBxTW1. This was an unexpected result since the bulky naphthalene molecule might not have been expected to fit in enzyme active site. These data prompted us to test for the formation of a new xyloside by using 2,6-DHN as an acceptor, due to the existing structural similarities between this compound and 1- and 2-naphtol, and because the potential transxylosylation product would be 2-(6-hydroxynaphthyl) β-D-xylopyranoside, a well known antiproliferative compound. The three compounds contain a naphthalene ring which is monohydroxylated in the case of 1- and 2-naphtol and dihidroxylated for 2,6-DHN. The absence of any other significant difference led us to expect that this compound would also work as transxylosylation acceptor for rBxTW1. The desired xyloside, 2-(6-hydroxynaphthyl) β-D-xylopyranoside, functions as selective inhibitor of transformed or tumor-derived cell growth by acting as an alternative primer for heparan sulfate synthesis. These interesting properties had been widely demonstrated *in vitro* by adding the compound to cultures of normal and transformed cell lines such as HFL-1 cells (human fetal lung fibroblasts), 3T3 A31 cells (mouse 3T3 fibroblasts) and T24 cells (human

bladder carcinoma cells) and *in vivo* with a subcutaneous tumor model in mice (Jacobsson et al., 2006; Mani et al., 2004).

Since the selected library did not include 2,6-DHN, the role of this compound as acceptor for rBxTW1 was evaluated by the direct detection of the new product in TLC, which is a better approach when assaying single compounds. With the set of conditions initially fixed for the transxylosylation assay, a faint new spot, probably corresponding to the reaction product, was observed upon developing the TLC plate (data not shown). HPLC analysis of the reaction mixture allowed estimation of the concentration of the hypothetical new xyloside to be 0.19 mM. The reaction conditions were further optimized using a response surface method, specifically BBD, in order to enhance the production of 2-(6-hydroxynaphthyl) β-D-xylopyranoside. The matrix of the experiments generated by the BBD approach and the outcomes from this analysis are collected in Table 4.7.

Table 4.7. Box–Behnken experimental design for optimization of 2-(6-hydroxynaphthyl)-β-D-xylopyranoside.

[2,6-DHN] (g/L)	Temperature (°C)	[Xylobiose] (mM)	time (min)	Enzyme (g/L)	pH	[Product] (mM)
0.30	40	60	10	0.055	4.1	0.04
3.00	40	60	10	0.055	4.1	0.45
0.30	60	60	10	0.055	4.1	0.05
3.00	60	60	10	0.055	4.1	0.37
0.30	40	60	60	0.055	4.1	0.13
3.00	40	60	60	0.055	4.1	1.09
0.30	60	60	60	0.055	4.1	0.12
3.00	60	60	60	0.055	4.1	0.55
1.65	40	20	35	0.010	4.1	0.13
1.65	60	20	35	0.010	4.1	0.05
1.65	40	100	35	0.010	4.1	0.13
1.65	60	100	35	0.010	4.1	0.07
1.65	40	20	35	0.100	4.1	0.34
1.65	60	20	35	0.100	4.1	0.41
1.65	40	100	35	0.100	4.1	0.80
1.65	60	100	35	0.100	4.1	0.83
1.65	50	20	10	0.055	2.2	0.23
1.65	50	100	10	0.055	2.2	0.11
1.65	50	20	60	0.055	2.2	0.37
1.65	50	100	60	0.055	2.2	0.55
1.65	50	20	10	0.055	6.0	0.26
1.65	50	100	10	0.055	6.0	0.29

Table 4.7 continued

1.65	50	20	60	0.055	6.0	0.48
1.65	50	100	60	0.055	6.0	0.80
0.30	50	60	10	0.010	4.1	0.00
3.00	50	60	10	0.010	4.1	0.07
0.30	50	60	60	0.010	4.1	0.05
3.00	50	60	60	0.010	4.1	0.31
0.30	50	60	10	0.100	4.1	0.09
3.00	50	60	10	0.100	4.1	0.85
0.30	50	60	60	0.100	4.1	0.11
3.00	50	60	60	0.100	4.1	1.31
1.65	40	60	35	0.010	2.2	0.18
1.65	60	60	35	0.010	2.2	0.00
1.65	40	60	35	0.100	2.2	0.67
1.65	60	60	35	0.100	2.2	0.19
1.65	40	60	35	0.010	6.0	0.13
1.65	60	60	35	0.010	6.0	0.02
1.65	40	60	35	0.100	6.0	0.77
1.65	60	60	35	0.100	6.0	0.49
0.30	50	20	35	0.055	2.2	0.05
3.00	50	20	35	0.055	2.2	0.49
0.30	50	100	35	0.055	2.2	0.10
3.00	50	100	35	0.055	2.2	0.66
0.30	50	20	35	0.055	6.0	0.06
3.00	50	20	35	0.055	6.0	0.66
0.30	50	100	35	0.055	6.0	0.11
3.00	50	100	35	0.055	6.0	0.67
1.65	50	60	35	0.055	4.1	0.68
1.65	50	60	35	0.055	4.1	0.69
1.65	50	60	35	0.055	4.1	0.67
1.65	50	60	35	0.055	4.1	0.67
1.65	50	60	35	0.055	4.1	0.70
1.65	50	60	35	0.055	4.1	0.65
1.65	50	60	35	0.055	4.1	0.67
1.65	50	60	35	0.055	4.1	0.66
1.65	50	60	35	0.055	4.1	0.66
1.65	50	60	35	0.055	4.1	0.68
1.65	50	60	35	0.055	4.1	0.66

The BBD matrix with the production data was analyzed by Design-Expert® software and fitted to the following quadratic model equation:

$$[\text{Product}] = -3.82 + 0.395A + 0.128B + 1.41\times10^{-3}C + 1.51\times10^{-2}D + 1.12E + 0.144F - 5.69\times10^{-3}AB + 2.06\times10^{-4}AC + 2.38\times10^{-3}AD + 3.32AE + 7.58\times10^{-3}AF - 5.46\times10^{-6}BC - 2.36\times10^{-4}BD - 2.88\times10^{-2}BE + 1.77\times10^{-3}BF + 7.48\times10^{-5}CD + 5.99\times10^{-2}CE + 1.13\times10^{-4}CF + 1.90\times10^{-2}DE + 3.72\times10^{-4}DF + 0.629EF - 6.54\times10^{-2}A^2 - 1.23\times10^{-3}B^2 - 5.16\times10^{-5}C^2 - 1.31\times10^{-4}D^2 - 60.8E^2 - 3.34\times10^{-2}F^2$$

A: [2,6-DHN] (g/L); B: Temperature (°C); C: [Xylobiose] (mM); D: Time (min); E: [Enzyme] (g/L); F: pH

The model predicted the production of the new xyloside as a function of the concentrations of 2,6-DHN and xylobiose, the amount of added enzyme, the temperature and time of reaction and the pH value. The analysis of variance test calculated by the software (Table 4.8) validated that the model matched the experimental data.

Table 4.8. ANOVA report from the quadratic model for xyloside production.

Source	Sum of Squares	df	Mean Square	F Value	p-value prob>F[a]
Model	5.59	27	0.21	32.28	< 0.0001
Residual	0.21	32	6.42×10^{-3}		
Lack of Fit	0.20	21	9.67×10^{-3}	48.72	< 0.0001
Pure Error	2.18×10^{-3}	11	1.99×10^{-4}		
Cor Total	5.59	27	0.21	32.28	< 0.0001

[a]Values of Prob > F less than 0.0500 indicate model terms are significant.

The Design-Expert® software was used to find the experimental conditions for maximal production predicted by the model within the selected limits for each parameter. The software generated a maximum value of 1.6 mM, which required a reaction mix composed of 3 g/L 2,6-DHN; 100 mM xylobiose; 0.1 g/L rBxTW1 and 50 mM sodium acetate pH 4.8, a temperature of 40.8 °C and a reaction time of 60 min. Under these conditions, the measured product concentration fit the theoretical value of 1.6 mM. Therefore, application of the response surface model enabled an 8-fold increase (from 0.19 mM to 1.6 mM) in xyloside production.

The reaction parameters from the former Box-Behnken adjustment were further changed according to the availability of reactants and enzyme in order to produce hypothetical product 2-(6-hydroxynaphthyl) β-D-xylopyranoside for its purification and identification. Since pure commercial xylobiose is an expensive substrate, but the enzyme is easily produced and purified, a new estimation was run by the model, decreasing xylobiose concentration from 100 to 50 mM, while no limits were set on enzyme concentration, temperature and reaction time. With these new

settings, the predicted optimal conditions included higher enzyme concentration and longer reaction time (0.1 to 0.15 g/L and 60 to 80 min, respectively), while temperature was slightly lower to delay enzyme inactivation and pH changed from 4.8 to 5.5. Under these conditions, a maximum concentration of 1.5 mM was predicted, and an actual value of 1.4 mM was obtained in this assay. This value is close to the 1.6 mM achieved when no limits were applied to xylobiose concentration. In both cases, the empirical values corroborated the theoretical predicted data.

The reaction mix was concentrated and analyzed by semi-preparative HPLC, purifying a major product peak and a minor one (Product 2) that was not previously detected in analytical-scale reactions. The yield of this by-product was, however, very low (0.2 mM). Product 2 eluted during the acetonitrile gradient, before the hypothetical 2-(6-hydroxynaphthyl) β-D-xylopyranoside (Product 1), suggesting that Product 2 may have incorporated a second xylose unit and thereby acquired increased polarity. If this assumption is true, the most probable scenarios are a second xylose unit attached either to the remaining free hydroxyl group of the 2-(6-hydroxynaphthyl) β-D-xylopyranoside or to the xylose already present. As the demonstrated regioselectivity of the native enzyme (Chapter I) is expected to persist in the recombinant form, the latter option may occur through a β(1→4) linkage, converting the xylose substituent into xylobiose. For complete identification of products, they were analyzed by NMR as follows.

4.2.6. Structural elucidation of the 2,6-DHN transxylosilation products by NMR

Analysis of ^{1}H and ^{13}C-NMR experiments of the two purified products allowed the elucidation of their structure. As expected, Product 1 was found to be 2-(6-hydroxynaphthyl) β-D-xylopyranoside (Fig. 4.15A, Table 4.9) in accordance with data described for the chemically synthesize compound (Siegbahn et al., 2011) while Product 2, where two different set of xylose signals appeared, was identified as 2-(6-hydroxynaphthyl) β-D-xylobioside (Fig. 4.15B, Table 4.9). The synthesis of the latter product instead of 2,6-dihydroxynaphthalene bis(β-D-xylopyranoside), that due to symmetry of the molecule would have only one set of xylose signals (Johnsson et al., 2007), indicated the preference of the enzyme for the sugar hydroxyl over the second naphthyl alcohol.

As far as we know the synthesis of 2-(6-hydroxynaphthyl) β-D-xylobioside has not been reported previously. The chemical synthesis of this compound would be more challenging than that of the xyloside because of the need to attach the second xylose moiety specifically to the C-4 hydroxyl group of the acceptor sugar (Danby and Withers, 2016). The potential anti-proliferative role of 2-(6-hydroxynaphthyl)-β-D-xylobioside

has not yet been investigated and merits attention. Further research using new and cheaper xylose donor will be necessary in order to carry out the required increase of production for the anti-proliferative analyses.

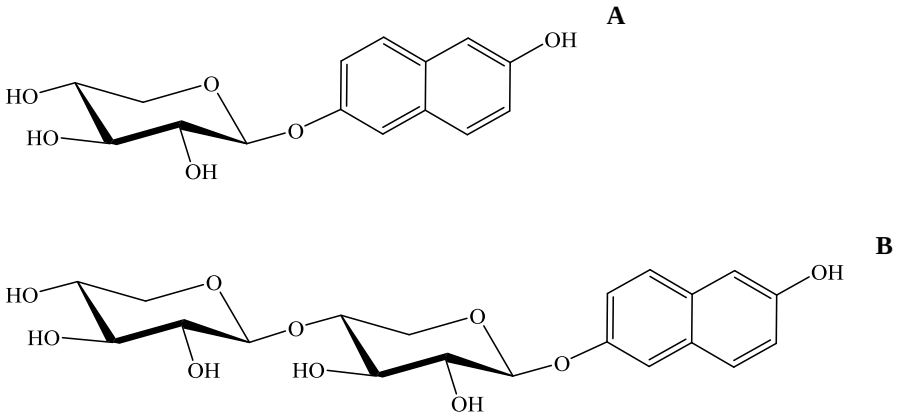


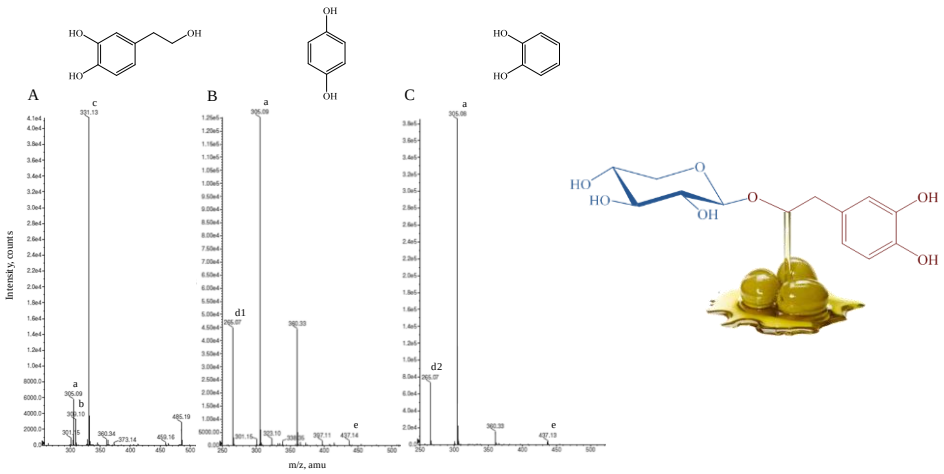
Fig. 4.15. (A) 2-(6-hydroxynaphthyl) β-D-xylopyranoside (Product 1) and (B) 2-(6-hydroxynaphthyl) β-D-xylobioside (Product 2) synthesized by rBxTW1 catalyzed transxylosylation. (A) Product 1 is formed in one step when a xylose moiety is attached to an hydroxyl group of 2,6-DHN. (B) The attachment of a second xylose to the former one by a β(1→4) linkage converts Product 1 into Product 2.

Table 4.9. Chemical shift data from 2-(6-hydroxynaphthyl) β-D-xylopyranoside and 2-(6-hydroxynaphthyl) β-D-xylobioside.

	2-(6-hydroxynaphthyl) β-D-xylopyranoside		**2-(6-hydroxynaphthyl) β-D-xylobioside**	
	^{1}H (ppm)	**^{13}C (ppm)**	**^{1}H (ppm)**	**^{13}C (ppm)**
H1'			4.54	102.14
H2'			3.33	73.05
H3'	-	-	3.49	75.88
H4'			3.69	69.50
H5'			3.37	65.50
H5'			4.04	
H1	5.22	101.35	5.24	101.27
H2	3.65	73.17	3.69	72.98
H3	3.64	75.79	3.76	73.95
H4	3.78	69.39	3.94	76.60
H5	3.56	65.50	3.64	63.13
H5	4.10		4.23	
	7.24	119.21	7.24	119.21
	7.31	109.64	7.31	109.64
Naphthalene ring	7.34	119.59	7.34	119.59
	7.53	111.78	7.53	111.78
	7.82	128.49	7.82	128.49
	7.84	129.27	7.84	129.27

4.3. CHAPTER III

REGIOSELECTIVE GLYCOSYLATION OF PHENOLIC ANTIOXIDANTS BY A β-XYLOSIDASE FROM *Talaromyces amestolkiae*: A NOVEL HYDROXYTYROSOL XYLOSIDE DISPLAYING HIGH NEUROPROTECTIVE EFFECT



4.3.1. Background

Many phenolic antioxidants are used as food additives in order to preserve the organoleptic properties of alimentary products because they delay the oxidation processes on proteins and lipids. Moreover, these compounds display well-known health benefits so they are added to functional foods and dietary supplements (Choe and Min, 2009; Chun et al., 2010). However, there are some drawbacks limiting their application: the most important ones are instability as well as low solubility and permeability which lead to poor bioavailability (Ratnam, V et al., 2006).

Olive oil is one of the main commodities of the Mediterranean agro-industry and, as a consequence, olive production in Southern Europe amounted 9 millions of tons in 2014 (FAOSTAT). Taking into account that yields are about 8 L of oil per 100 kg of olives, a considerable quantity of residual biomass is annually generated. These by-products display high concentration of phenolic compounds which implies toxicity concerns, but also an invaluable source of antioxidants (Daassi et al., 2014; Romero-Garcia et al., 2014). Among them, 3,4-dihydroxyphenylethanol, commonly known as hydroxytyrosol (HT) and also present in olive oil, is the most potent displaying strong anti-inflammatory and neuroprotective properties (Gonzalez-Correa et al., 2008; Richard et al., 2011). In recent years several studies have demonstrated the effect of this antioxidant on the prevention of cardiovascular diseases, cancer (Granados-Principal et al., 2010) and other chronic diseases (Lee-Huang et al., 2007; Schaffer et al., 2007).

HT is well absorbed in a dose-dependent manner, but it displays a poor bioavailability because of its rapid conversion into its sulphate and glucuronide metabolites by first-pass metabolism (Medina et al., 2010). The glycosylation of phenolic antioxidants has emerged as a way to improve their bioavailability, stability or other properties of interest. Synthetic chemical approaches imply many steps (Medina et al., 2010; Shimotori et al., 2012), low specificity and toxic side products, but enzymatic glycosylation by using glycosidases or glycosyltransferases is an eco-friendly and highly specific alternative (Mathew and Adlercreutz, 2013; Torres et al., 2011). In the case of HT, considerable efforts have been done for *in vitro* synthesis or isolation of its physiological metabolites (Khymenets et al., 2010; Lucas et al., 2009), but there are few works about the production of non-natural glycosides of HT. These new glycosides may simplify the study of its role *in vivo* or display enhanced properties, becoming more attractive from the industrial perspective (Trincone et al., 2012).

The aim of this work was to develop a one-step method to enzymatically synthesize non-natural glycosides of polyphenolic antioxidants with commercial interest. The GH3 β-xylosidase BxTW1 from

T. amestolkiae heterologously expressed in *P. pastoris* was used as catalyst. After the screening of the selected antioxidants, this chapter reports the regioselective and efficient xylosylation of HT, as well as positive results for catechol and hydroquinone. The novel xyloside from HT obtained in this work has been evaluated in order to determine its anti-inflammatory and neuroprotective properties.

4.3.2. Test of rBxTW1 as a tool for transxylosylation of polyphenolic antioxidants

Previous reports on BxTW1 revealed that this enzyme produced high transxylosylation yields using alkan-n-ols, sugar alcohols and monosaccharides as acceptors in a regioselective way (Chapter I). However, glycosidases were already known for their ability to glycosylate sugars or alcohols including most of the tested compounds in that work (Crout and Vic, 1998; Draelos, 2007). Previously (Chapter II), the transxylosylation of xylobiose with 2,6-hydroxynaphtalen was achieved, obtaining the anti-tumorigenic 2-(6-hydroxynaphthyl) β-D-xylopyranoside with a modest yield of 0.47 g/L. In order to demonstrate the wide acceptor versatility of rBxTW1 a further high-throughput analysis was performed. Attending to these capacities, the enzyme was considered a potential candidate for glycosylating polyphenolic antioxidants, although the presence of bulky aromatic rings and their high insolubility were supposed to decrease transxylosylation. In this sense, hydroxytyrosol, apart from its biological properties, was a promising antioxidant to assay since it is formed by an aliphatic chain with a primary alcohol group, which was expected to be a suitable target for being efficiently glycosylated by rBxTW1. In addition, due to the demonstrated acceptor versatility of this enzyme, the screening was extended to other potential phenolic acceptors naturally found in plant biomass and with commercial interest: resveratrol, quercetin, (±)-α-tocopherol, hesperetin, hesperidin, gallic acid, L-ascorbic acid, ECGC, ferulic acid, HQ and catechol.

In order to test transxylosylation, a reaction was carried out using xylobiose and HT as donor and acceptor respectively, in the conditions described above. The optimum pH for rBxTW1 is pH 3.0, which enhanced the stability of HT or any other phenolic antioxidants, prevented self-oxidation, and allowed long reaction times and long-term storage of the potential new xylosides. The reaction mixtures were tested by TLC, appearing the hypothetical new xyloside as a new spot (data not shown).

After a first screening, HQ and catechol emerged as positive hits in addition to the expected HT. Figure 4.16 displays the structures of these phenolic derivatives. No glycosylated derivatives were detected with the rest of acceptors.

The reaction mixtures obtained with HT, HQ and catechol were analyzed by ESI-MS. The presence of a mono-xylosylated derivative of the acceptor was confirmed for the three compounds (Fig. 4.17). In addition, xylotriose was also detected in the reactions with HQ and catechol, indicating that part of the xylobiose was used as acceptor (Chapter I).

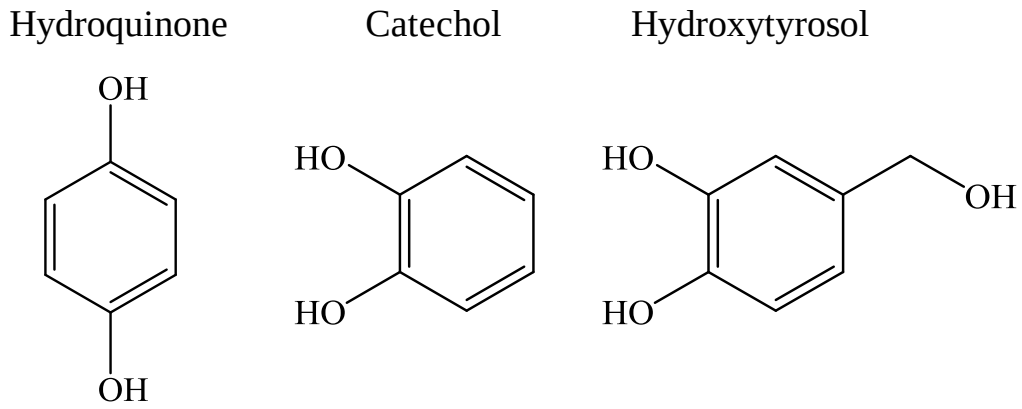


Fig. 4.16. Positive acceptors for transxylosylation with rBxTW1.

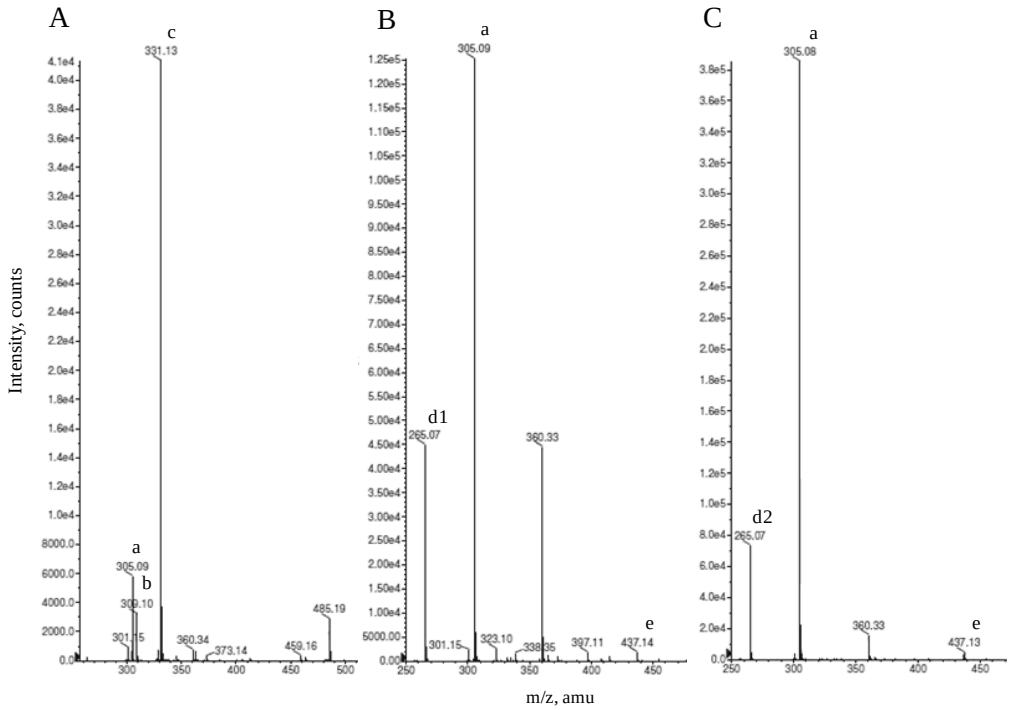


Fig. 4.17. Mass spectra of a transxylosylation reaction mixture with hydroxytyrosol (A), hydroquinone (B) and catechol (C). The identified adducts are indicated, a: Xylobiose+Na^+; b: HT-Xyloside+Na^+; c: 2HT+Na^+; d1: HQ-xyloside+Na^+; d2: Catechol-xyloside+Na^+; e: Xylotriose+Na^+.

Hydroquinone and catechol are well-known phenolic antioxidants. HQ is the most used skin whitening for its role as tyrosinase inhibitor, but there is a rising concern about its safety on human health and industry is looking for safer and active hydroquinone derivatives (Draelos, 2007). A considerable effort has been made on the synthesis of α-arbutin

(hydroquinone-*O*-α-glucoside) (Mathew and Adlercreutz, 2013; Seo et al., 2012), but the search of new glycosides with different properties continues (Kang et al., 2009; Sugimoto et al., 2005). The hydroquinone xyloside has been reported as an alternative for inhibiting tyrosinase (Chiku et al., 2009), so it constitutes an interesting compound to be synthesized by rBxTW1.

Regarding catechol, it is the core of many compounds used as drugs in the treatment of bronchial asthma, hypertension, Parkinson's disease, myocardial infarction or even HIV (Bollini et al., 2011; Nagaraja et al., 2001). Catechol glycosides, as the catechol xyloside reported in this work, may lead to novel drugs with improved functionalities. Indeed, some esters of catechol glycosides have been studied due to their antihyperglycemic activity (Kumar et al., 2009). The enzymatic synthesis of catechol xyloside was previously reported by Chiku *et al*. (2008) using a xylanase, but the process produced a mixture of glycoconjugates with one to four units of xylose.

4.3.3. Reaction specificity

Upon preliminary analysis of the reactions by TLC, the positive hits were analyzed by HPLC for a deeper insight of the regio- and stereo-specificity in transxylosylation of these compounds catalyzed by rBxTW1. Different mixtures of methanol and water were employed as mobile phases in order to get the best separation. Surprisingly, peaks corresponding to the catechol-xylosides were not detected, which could be probably attributed to a low concentration of the xyloside formed.

The number of HT- and HQ-xylosides was determined by HPLC. Compounds containing an aromatic ring (derived from HQ or HT) were detected by measuring the absorbance at 241 and 268 nm, and hence remaining xylobiose, xylose or other side products did not interfere with the analysis. Chromatograms displayed a single new peak for both acceptors, thus confirming monoglycosylation. These results suggested that the transxylosylation catalyzed by rBxTW1 was chemo- and regioselective.

4.3.4. Optimization of transxylosylation

The influence of the concentration of donor and acceptor together with the capacity of the enzyme to hydrolyze the synthesized xylosides were studied in order to find optimal reaction conditions. The concentration of the xyloside under the different experimental conditions (see Table 3.1) was determined by HPLC.

Assays using HT as acceptor were carried out with 18 U/mL of rBxTW1. In order to study the influence of HT concentration in the production, the initial concentration of xylobiose was fixed at 40 mM and the formation of the HTX was analyzed along time using 75-600 mM HT

(Fig. 4.18A). Then, the HT concentration that gave rise to the highest production of the xyloside was selected for assessing a range of concentrations of xylobiose (Fig. 4.18B).

Figure 4.18A clearly shows that maximal production of the xyloside was quickly achieved at all concentrations of HT tested. However, with 75 and 150 mM HT a dramatic decrease of the product occurred beyond the first 10 min. This suggests that the HT-xyloside is also a substrate for rBxTW1, which starts its hydrolysis when xylobiose and the xyloside are available at similar concentrations. On the contrary, with 300 and 600 mM HT in the reaction medium the content of xyloside seemed quite stable after reaching the maximum, at least during 4 hours. These high concentrations of HT displace the ratio hydrolysis/transxylosylation towards synthesis, since HTX instead of being hydrolyzed can act as donor of a new transxylosylation reaction, attaching the xylosyl moiety to another molecule of HT and by this way maintaining stable the overall concentration of the product. The diminished production level reached with 600 mM HT in comparison to 300 mM could be due to substrate inhibition.

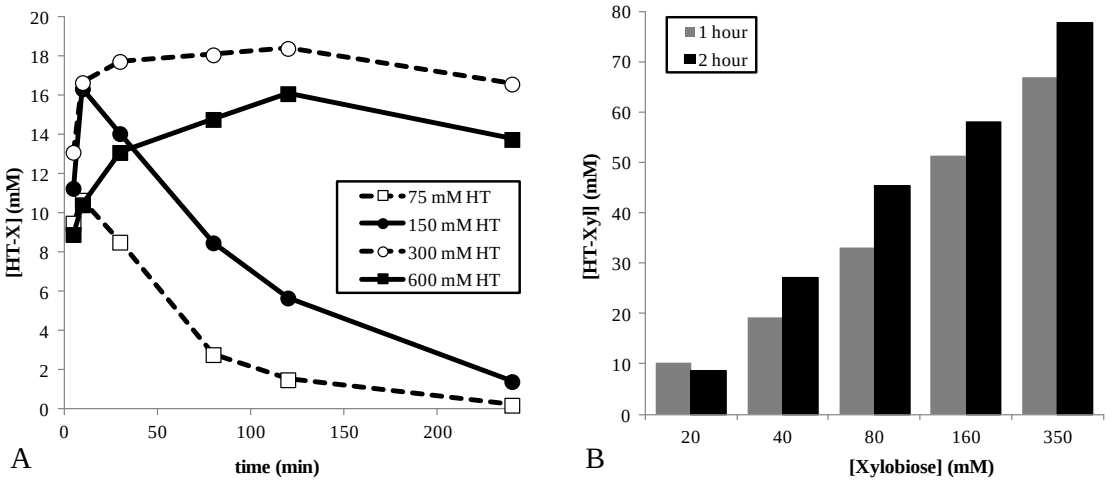


Fig. 4.18. Optimization of acceptor (A) and donor (B) concentration for the synthesis of HTX. Reactions were performed at pH 3.0 and 50 ºC. (A) Time course of the HT-xyloside production varying the concentration of HT. Initial concentration of xylobiose was fixed as 40 mM. (B) Relation between xyloside production and initial xylobiose. Reactions contained 300 mM HT as acceptor.

Figure 4.18B illustrates the increase of the xyloside production with the increasing donor concentration, fixing the initial acceptor concentration at 300 mM. From 20 to 40 mM xylobiose the xyloside almost doubled its concentration, but from 160 to 350 mM the increase was less significant, only a 30%. This may be caused by the competition between HT and xylobiose, because the disaccharide also acts as an efficient acceptor for rBxTW1, as demonstrated by the synthesis of xylotriose as co-product (Fig. 4.17). It is also noticeable that in reactions with 20 mM xylobiose the

amount of product detected between 1 and 2 h was quite stable, which could be due to the rapid consumption of the available donor.

According to the definitions above, the yield describes the molar relation between product and acceptor. Among the conditions assayed, the best result (26.6% yield) was attained in 2 h with 300 mM HT and 350 mM xylobiose, yielding 77.7 mM of HT-xyloside (22.2 g/L). This production is the highest reported for a non-natural hydroxytyrosol glycoside synthesized by transglycosylation. Trincone *et al*. (2012) reported 45% conversion studying the glucosylation of HT with the α-glucosidase from *Aplysia fasciata*; however, they used a small concentration of HT (5 mM) and a high excess (30:1) of maltose. In addition, the process was neither chemo- nor regioselectivity, giving rise to three glucosides in the aliphatic alcohol and the two phenolic hydroxyls, respectively. On the contrary, rBxTW1 catalyzes the synthesis, at a high concentration level, of one single xyloside, which simplifies product purification and reaction control.

The effect of the concentration of HQ on the reaction yield was also studied by maintaining the initial concentration of xylobiose at 40 mM. Preliminary assays (data not shown) revealed a much rapid formation and hydrolysis of the xyloside, so the reaction conditions were adjusted by decreasing the enzyme concentration to 0.9 U/mL (Table 3.1). The formation of HQ-xyloside (HQX) was analyzed along a range of concentrations of HQ and different times. An inhibitory effect of this acceptor was also observed at the maximum concentration assayed (data not shown). The maximal value obtained for 110 mM HQ at 1 h and for 225 mM HQ at 2 h was virtually the same (Fig. 4.19A). Then, a concentration of 110 mM was selected as the optimal, as the highest yield was reached in less time and with less acceptor, and used for assaying a range of concentrations of xylobiose (Fig 4.19B).

Figure 4.18B displays a remarkable increase of HQX production with low concentrations of xylobiose, and a modest change with the higher values, as occurred with HT. In fact, the HQX production is higher using 160 mM xylobiose than with 350 mM. As indicated above for HT, we propose that xylobiose and HQ compete as acceptors of transxylosylation although in this case the effect was more evident. The data presented in Figures 4.18 and 4.19 suggest that HT is a preferred acceptor for rBxTW1 in comparison to HQ, though the optimal concentrations for both acceptors are notably different.

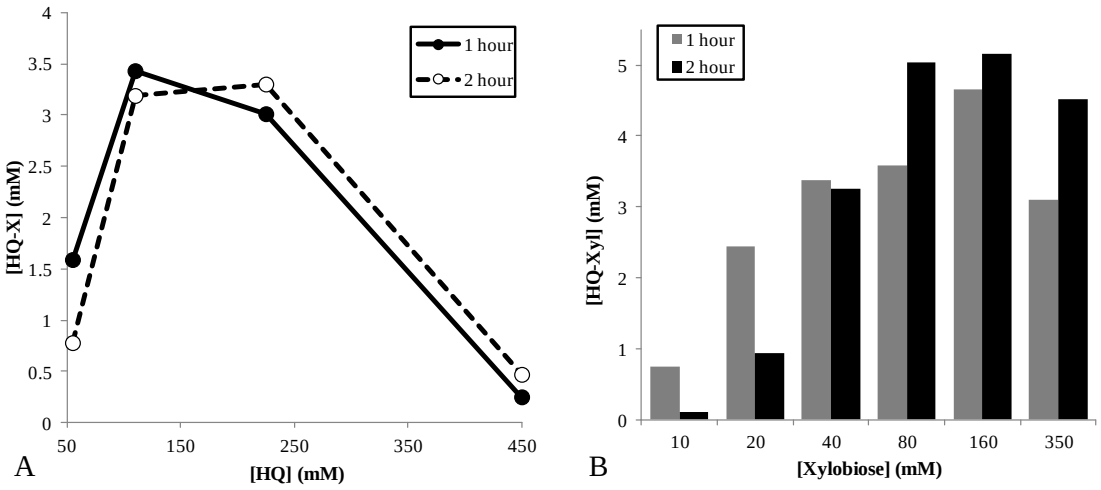


Fig. 4.19. Optimization of acceptor (A) and donor (B) concentration for the synthesis of HQX. Reactions were performed at pH 3.0 and 50 ºC. (A) Time course of the HQ-xyloside production using 110 mM HQ and 40 mM xylobiose. (B) Relation between xyloside production and initial xylobiose by using the optimum concentration of HQ (110 mM).

The comparison between the two reaction times (Fig. 4.19B) demonstrated higher concentrations of xyloside after 1 h at 10, 20 and 40 mM xylobiose, probably due to hydrolysis of the product at longer reaction times once the maximum concentration was reached. This was also observed for HT but it is especially important in the case of HQ, because the hypothetically synthesized xyloside is chemically related to 4-nitrophenyl-β-D-xylopyranoside, and rBxTW1 displayed a higher k_{cat}/K_m against *p*NPX than against xylobiose (Chapter II), so the enzyme may hydrolyze the new xyloside preferentially even in the presence of a higher concentrations of the donor.

The highest yield achieved for this reaction (4.7%) was obtained using 110 mM HQ and 160 mM xylobiose for 2 h of reaction. In these conditions 5.2 mM (1.2 g/L) of HQ-xyloside was obtained, a value similar to those reported for other transglycosylation products of HQ (Kang et al., 2009; Prodanovic et al., 2005) but far away from the high levels reported by Seo *et al*. (2012).

4.3.5. Purification and identification of HTX by NMR

Since HTX was produced at higher yields than HQX, it was selected for a scale-up of its synthesis in order to confirm its structure by NMR and to assay its biological properties. The HTX produced from 4 mL-reactions was purified by semipreparative HPLC as described above, obtaining 27.3 mg of pure xyloside.

The signals from each element of HTX were identified and assigned by employing ^{1}H, COSY and HSQC-edited experiments. The position of the glycosylation was unequivocally determined by the existence of a

strong NOE between the anomeric H_1 proton from the xylose residue and the protons of the aglycon element that is linked to it. In this particular case, the CH_2 protons designed as "a" in Figure 4.20 and Table 4.10 were clearly identified.

The reaction product was identified as 3,4-dihydroxyphenyl-ethyl-*O*-β-D-xylopyranoside. To the best of our knowledge, the product synthesized by rBxTW1 is a novel compound. This result confirmed that this enzyme was catalyzing the synthesis of a single product in a chemo- and regioselective way. The acceptor of transglycosylation was the primary alcohol, which supports the preference of rBxTW1 for the primary-alcohols as acceptors that was proposed previously (Chapter I) and also explained the higher yields obtained for HT in comparison with HQ, with no primary alcohols. The preference for the aliphatic OH compared with the phenolic hydroxyls could be also favored by the low pH (3.0) at which rBxTW1 displays it maximum activity. It is well reported that the relative reactivity between primary and aromatic OHs in glycosidase-catalyzed acceptor reactions is determined by the nucleophilicity and the pK_a of both hydroxyls (Mena-Arizmendi et al., 2011). Despite nucleophilicity of aliphatic OHs is higher, the lower pK_a of aromatic hydroxyls may revert the reactivity thus giving rise to glycosylated derivatives in the aromatic ring.

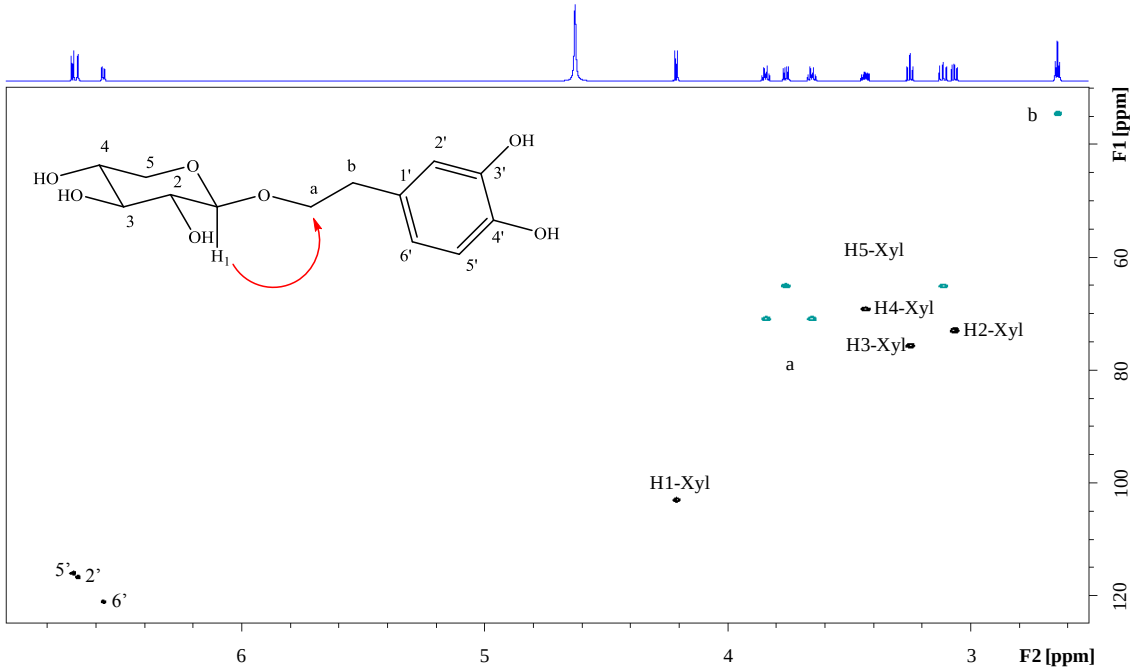


Fig. 4.20. DEPT-HSQC NMR spectrum of the 3,4-dihydroxyphenyl-ethyl-*O*-β-D-xylopyranoside compound, showing the assignation of all signals. The glycosylation site was determined from a 2D-NOESY spectrum which showed crosspeaks between the anomeric proton H1-Xyl and the CH2 (a) from the ethyl moiety (arrow). The product is formed in one step when a xylose moiety is attached to the primary hydroxyl group of HT by rBxTW1 catalyzed transxylosylation.

Table 4.10. Chemical shifts data from 3,4-dihydroxyphenyl-ethyl-*O*-β-D-xylopyranoside.

	^{1}H (ppm)	^{13}C (ppm)
Aryl-H6'	6.57	120.94
Aryl-H2'	6.67	116.57
Aryl-H5'	6.69	115.92
H1-Xylose	4.21	102.82
H3-Xylose	3.25	75.63
H2-Xylose	3.06	72.88
CH_2(a)α	3.65	70.78
CH_2(a)β	3.84	
H4-Xylose	3.43	69.00
H5-Xyloseα	3.11	64.96
H5-Xyloseβ	3.76	64.96
CH_2(b)	2.64	34.38

In this work, the low pH of the process limited the reactivity of phenolic OHs of HT, although structural features in the active-site environment could also contribute to the notable chemo- and regiospecificity displayed by rBxTW1.

4.3.6. Biological activity of 3,4-dihydroxyphenyl-ethyl-*O*-β-D-xylopyranoside (HTX)

Neuroprotection and anti-inflammation are well-known effects of HT itself (Gonzalez-Correa et al., 2008; Richard et al., 2011), therefore the assays focused on comparing its effects with those produced by HTX, HTA and resveratrol.

First, cell viability in the presence of the tested substances was evaluated by addition of HTX, HT, HTA and RES to RAW 264.7 macrophage and SH-SY5Y neuroblastoma cultures. The viability of macrophages (Fig. 4.21A) decreased for the 1% DMSO control and for every antioxidant at 1 and 10 μM. On the contrary, when their concentration increased to 100 μM about 100% viability was restored for HTX, HT and HTA whereas RES induced a remarkable viability decrease to 44%, which indicated certain cytotoxicity of this compound. In neuron cultures (Fig. 4.21C), addition of DMSO slightly decreased cell viability, but it was completely restored using 1 or 10 μM antioxidants. Interestingly, 100 μM HTX and HTA increased notably cell viability, to values well above those of control cultures without DMSO, suggesting their beneficial effect on neurons even in the absence of an external stress. This effect was

not observed for HT that displayed a high viability at all concentrations. RES also showed toxicity in neurons at 100 μM concentration.

Once established the safety of HTX, its anti-inflammatory and neuroprotective activity was assayed and compared to that of the other compounds tested. The former assays were carried out in the presence of LPS as pro-inflammatory agent (Fig. 4.21B). As a general rule, all compounds produced an increase in cell viability between 120-160% regardless of the dose added to the cell culture. Only the treatment with 100 μM RES produced a marked loss of viability, which was not surprising attending to the results from cell viability tests.

However, the neuroprotective activity of the compounds evaluated was very different (Fig. 4.21D). RES displayed a slight neuroprotection from the oxidative damage at concentrations of 1 and 10 μM, whereas HT did not protect the cell culture. In contrast, the addition of 10 μM HTX or HTA produced neuroprotection, more remarkable for HTA, and at 100 μM both compounds led to complete neuroprotection.

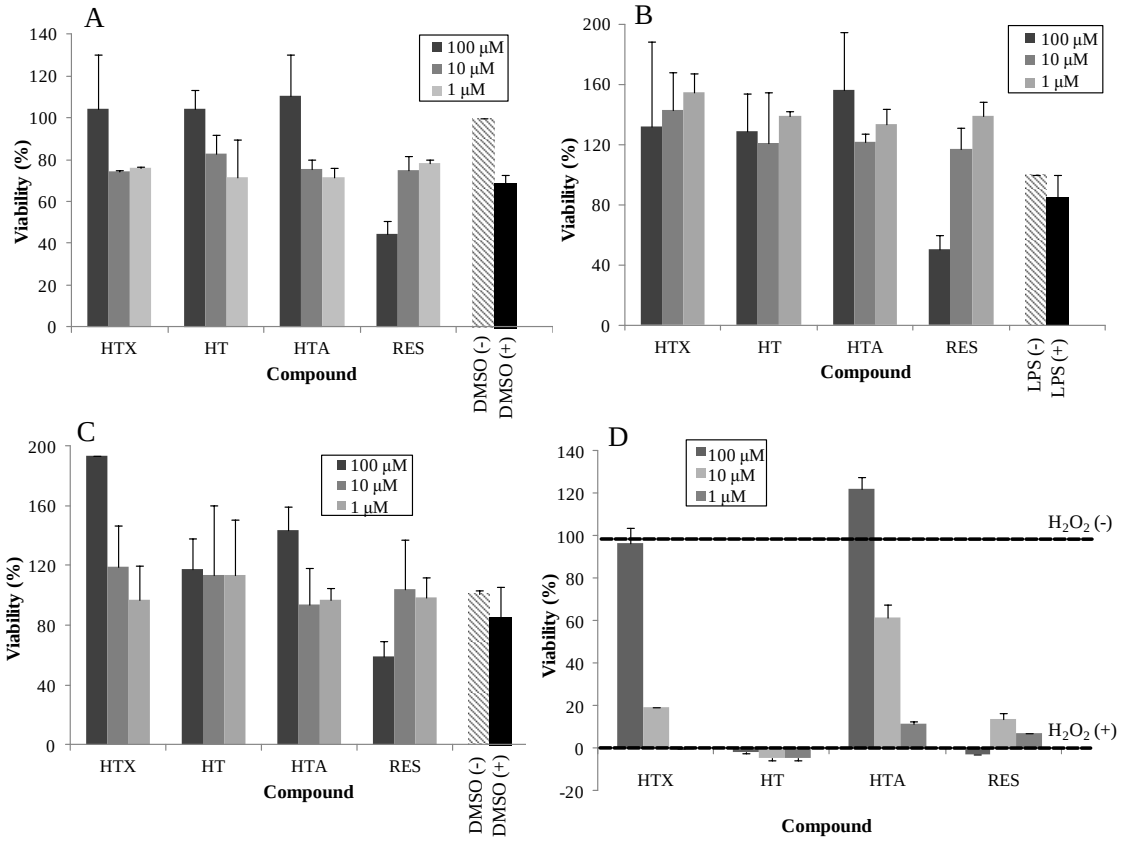


Fig. 4.21. Biological activity of 3,4-dihydroxyphenyl-ethyl-*O*-β-D-xylopyranoside: Cells viability (A, C), anti-inflammatory effect (B) and neuroprotection (D) were evaluated in the presence of HTX and three commercial antioxidants. Assays were performed on macrophage (A, B) and neuron (C, D) cell cultures. Controls with (+) and without (-) the stress agent were included in every case.

The absence of a protective effect in the case of HT may seem surprising since it has been reported as a strong neuroprotective agent. However, there are several methodological differences which make difficult the comparison between works and may explain the lack of neuroprotection in the conditions of the present study. Thereby, most of the *in vitro* experiments were carried out in rat brain slices inducing tissue damage by hypoxia (Cabrerizo et al., 2013; Gonzalez-Correa et al., 2008), sodium nitroprusside or Fe^{2+} (Schaffer et al., 2007) while the results displayed above were obtained in SH-SY5Y neuroblastoma cultures, a human derived cell line, and inducing oxidative damage with H_2O_2. There is also a recent report of Peng *et al*. (2015) assaying neuroprotection of HT in a neuroblastoma cell culture and also inducing oxidative damage by adding H_2O_2, but the selected-cell line was also from rat and they used a higher concentration of H_2O_2 (500 μM).

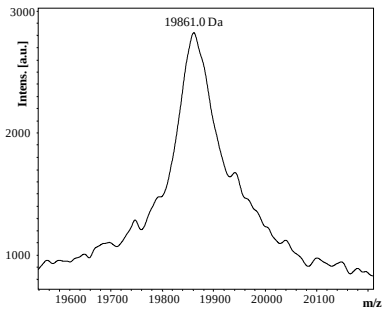
These assays indicated that in the tested conditions HTX is a safe antioxidant, which displays similar anti-inflammatory properties than HT itself, but remarkably higher neuroprotective effect. Regarding to HTA, it has been described as an acetyl-derivative of HT with increased lipophilicity, but keeping anti-inflammatory and neuroprotective qualities (Gonzalez-Correa et al., 2008; Lisete-Torres et al., 2012). The results from the current work are in good agreement with those reports, since HTA is much better neuroprotector than HT, as observed for HTX. It is worth highlighting that the differences in polarity between HTX and HTA may confer them specific fields of application. In this sense, and following the polar paradox (Frankel et al., 1994), more lipophilic derivatives, as HTA, display high antioxidant activity in lipid emulsions, whereas hydrophilic derivatives, as HTX, constitute more potent antioxidants in the case of oxidation of bulk lipids (Medina et al., 2009; Medina et al., 2010). In addition, the work of Saija *et al*. (1998) suggests that hydrophobic polyphenols are strong scavengers of chain-propagating lipid peroxyl radicals within the biological membranes whereas the hydrophilic counterparts, as HTX, act as scavengers of aqueous peroxyl radicals.

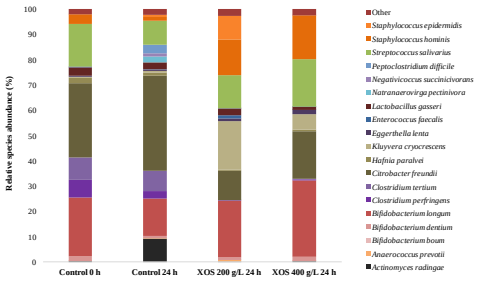
Besides the preliminary analysis of HTX biological properties, its cell uptake into RAW 264.7 macrophages and SH-SY5Y neuroblastoma cells also merits attention. Deglycosylation is a necessary step for cellular uptake of some polyphenolic glucosides (Scalbert and Williamson, 2000), but it seems unlikely that neurons or macrophages in cell cultures produce any enzyme displaying β-xylosidase activity capable of deglycosylating HTX. At the same time, the polarity of the xylose moiety is expected to prevent direct diffusion through cellular membranes (Scalbert and Williamson, 2000). These impairments suggest that HTX absorption could be related to the mechanisms for xylose uptake. Despite the information about pentoses uptake is very limited, it has been proposed that the glucose

transporter may play a role in xylose uptake (Rolston and Mathan, 1989). Taking this into account, it seems possible that the uptake of HTX is mediated by the same system, but further research is necessary to validate this hypothesis.

4.4. CHAPTER IV

PREBIOTIC EFFECT OF XYLOOLIGOSACCHARIDES PRODUCED FROM BIRCHWOOD XYLAN BY A NOVEL FUNGAL GH11 XYLANASE





4.4.1. Background

Xylooligosaccharides are soluble oligosaccharides derived from the incomplete hydrolysis of xylan. Interestingly, although the complete conversion of this heteropolysaccharide into xylose is a main target for bioethanol production, a huge interest is rising by XOS themselves. These oligosaccharides are composed of xylopyranose residues linked through β-1,4 bonds, with a degree of polymerization from 2 to 10 units, and a expanding number of studies about its production and purification are being carried out, since their potential as emerging prebiotics is becoming evident (Chapla et al., 2012; Immerzeel et al., 2014).

Gibson *et al*. (2004) defined prebiotics as "non-digestible (by the host) food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract". In this context, XOS have demonstrated their capacity to selectively stimulate the growth of probiotic microorganisms present in the lower gastrointestinal tract such as *Lactobacillus* and *Bifidobacterium* species (Aachary and Prapulla, 2011; Chapla et al., 2012). Besides this role as a classical prebiotic, XOS include other beneficial effects as possible antitumorigenicity, anti-inflammation and antialergy activity (Aachary and Prapulla, 2011). Together with these benefits, XOS lack of negative effects on human health or objectionable organoleptic properties (Aachary and Prapulla, 2009), which has made these oligosaccharides highly demanded as functional food ingredients.

In order to satisfy this demand, XOS are obtained from the xylan fraction of lignocellulosic materials by physicochemical or enzymatic methods. Physicochemical approaches mainly include autohydrolysis and acid hydrolysis. These processes are quite fast, but the substrate is partially converted into monosaccharides, reducing the production yield and releasing toxic by-products, making harder the subsequent purification phase. In contrast, the enzymatic approach is environmentally friendly, requires mild conditions, produces low monosaccharides' yields and non-toxic by-products (Chapla et al., 2012). The enzymatic hydrolysis of xylan is carried out by endoxylanases, which cut the xylan backbone releasing XOS (an extended description of this type of glycosidase is displayed in Introduction). Filamentous fungi are one of the most important microbial sources of both families and fungal xylanases are extracellular and display high activity in comparison with those from bacteria and yeasts (Polizeli et al., 2005). In this sense, the genera *Trichoderma* and *Aspergillus* have been extensively investigated in the search of cellulolytic and hemicellulolytic species. However, *Penicillium* strains also seem to be good candidates as sources of lignocellulolytic enzymes (Chavez et al., 2006).

In a previous study, a screening for lignocellulolytic fungi was carried out and *T. amestolkiae*, was selected for encoding a large amount of cellulases and hemicellulases (de Eugenio et al., 2017). The work presented

here reports the production, isolation and biochemical characterization of a new endoxylanase from this fungus. The capacity of the enzyme for converting xylan into xylooligosaccharides is evaluated and the prebiotic potential of the resultant XOS mixtures demonstrated.

4.4.2. XynM production

At laboratory scale it is usual to select a commercial xylan of high purity and homogeneity in order to induce xylanase activity, maximizing reproducibility and making results comparable with others from different sources (Chavez et al., 2006). For this reason, endoxylanase activity and total secreted proteins were measured in *T. amestolkiae* cultures with different commercial carbon sources. The endoxylanase-inducer effect of 1% glucose, 1% Avicel or 1%, 2% or 3% beechwood xylan in 6 day-old cultures was assayed (Fig. 4.22).

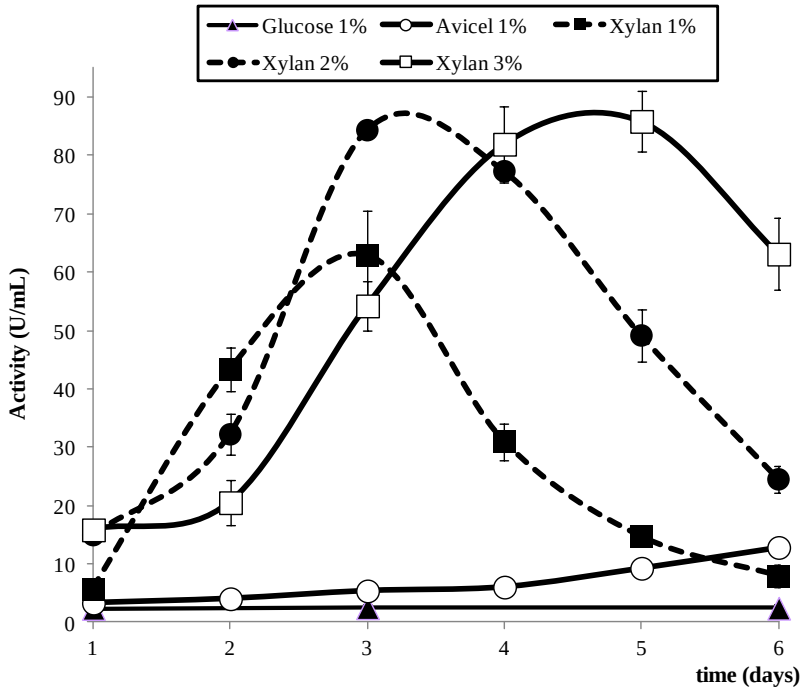


Fig. 4.22. Extracellular xylanase activity of *T. amestolkiae* cultures in Mandels medium in the presence of different carbon sources.

As expected, no endoxylanase activity was detected when glucose was the carbon source, since this monosaccharide has been extensively reported as a strong repressor of the hemicellulolytic metabolism in the genus *Penicillium* (Chavez et al., 2006). However, cultures with Avicel, a cellulosic substrate, produced a small endoxylanase induction (15% of the maximal activity). Induction of xylanolytic enzymes in fungi by this kind of substrates has already been reported suggesting an overlap between the expression pathways of cellulases and xylanases (Jørgensen et al., 2005). Cultures induced with 2% and 3% xylan showed the highest extracellular

endoxylanase activity, although maximal activity was achieved two days before with 2% xylan, which implies a more cost-effective enzyme production. Based on these results, 2% beechwood xylan was chosen as the best inducer for endoxylanase, which was produced, purified and characterized from these crude extracts.

4.4.3. Purification of XynM

Cultures were harvested at day 3 post-inoculation, (maximal activity), using crudes for enzyme isolation. Purification resulted in a final yield of 5.3% recovered activity. The pure enzyme (Fig. 4.23A) was stored at 4 ºC, maintaining its activity at least during six months. After the process, the specific activity increased from 61.4 to 118.3 U/mg, which represents a degree of purification of 1.9. These apparently low values may be understood taking into account that the initial endoxylanase activity was measured using the concentrated crudes against xylan, a complex polymer which needs the coordinate action of different enzymes to be completely hydrolyzed. Along the purification process, the progress in XynM isolation proceeded in parallel with a decrease in the auxiliary enzymes that participate in xylan hydrolysis, causing an unavoidable loss of activity that may explain the low final yield and degree of purification.

4.4.4. Characterization of XynM

The enzyme was determined to have an accurate molar mass of 19,861 Da (Fig. 4.23B) an isoelectric point around 5.5, and a monomeric active form. XynM displayed maximal activity at pH 3-4 and 50 °C and was stable across the wide range of pH assayed, although its thermostability was low at 50 °C and higher temperatures. These are typical properties previously reported for endoxylanases from *Penicillium* species (Chavez et al., 2006).

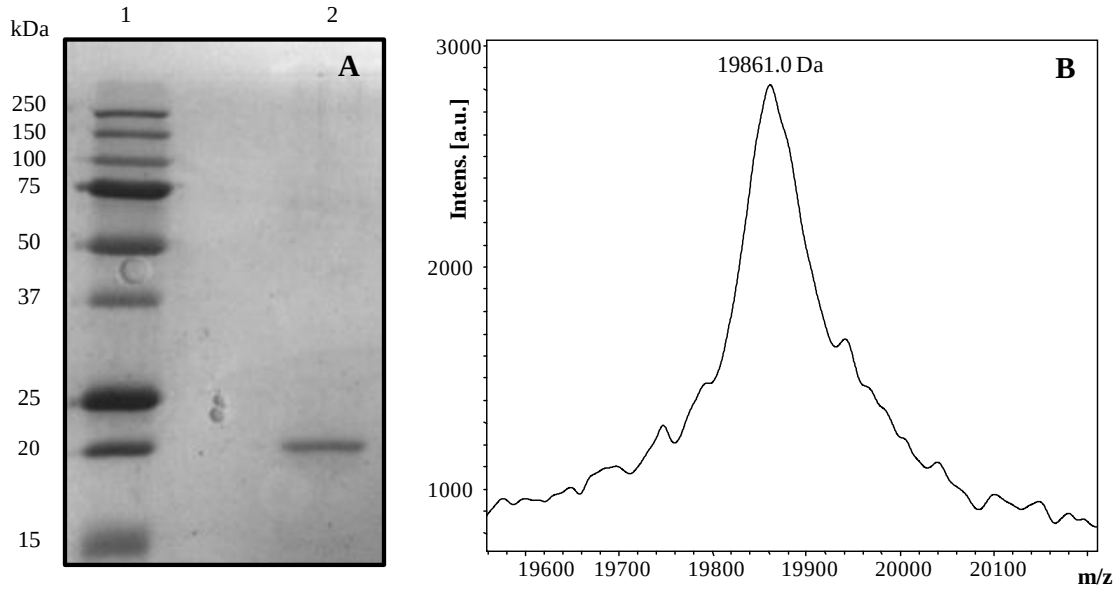


Fig. 4.23. A) SDS-PAGE analysis of pure XynM. B) Determination of the molecular mass of XynM by MALDI-TOF. Lanes: 1, molecular mass standards; 2, purified XynM. Intens., intensity; a.u., arbitrary units.

XynM displayed Michaelis-Menten kinetics against beechwood xylan, while it was inactive with the other tested substrates. This high specificity has been reported as a characteristic of GH11 xylanases (Polizeli et al., 2005). Its kinetic characterization revealed K_m and V_{max} values of 5.5±1.4 mg/mL and 129.0±12.6 U/mg, respectively, which are similar to those reported for several endoxylanases from *Penicillium* and *Talaromyces* species (Table 2). It should be noticed that some of the enzymes gathered in Table 2 displayed unusually high activities; this fact may be related to the method selected for protein quantification. The most remarkable cases were XYNC from *Penicillium funiculosum* and XynA from *Penicillium* sp.40, which displayed Vmax of 2,540 and 6,100 U/mg respectively, both of them quantified by the Bradford assay (Furniss et al., 2002; Kimura et al., 2000). Indeed, V_{max} value of XynM quantifying protein concentration by Bradford was 7,702 U/mg, suggesting that the BCA method may be a more accurate approach to quantify fungal xylanases.

It was the addition of some common chemical compounds to the reaction mix that revealed the most distinguishing characteristic of XynM: its high tolerance to Cu^{2+} and Hg^{+}. The case of Cu^{2+} is especially remarkable because it is commonly detected upon ash analysis of lignocellulosic biomass (Bin and Hongzhang, 2010). Both cations have been reported as strong inhibitors of endoxylanases (Belancic et al., 1995; Ryan et al., 2003). The obtained profiles of pH, temperature and effect of common chemical compounds are displayed in Figure 4.24.

Table 4.11. Comparison of the kinetic parameters determined for different fungal endoxylanases using several xylans as substrates.

Organism	Enzyme	Xylan	K_m (mg/mL)	V_{max} (U/mg)	Reference
Talaromyces amestolkiae	XynM	Beechwood	5.5	129.0[a]	This work
Talaromyces thermophilus	Xylanase	Birchwood	22.51	1.24	(Maalej et al., 2009)
Penicillium canescens	Xyl-31_{rec}	Glucuronoxylan Arabinoxylan	4.1 2.3	29.4 24.1	(Sinitsyna et al., 2003)
Penicillium capsulatum	XynA	Oat spelt	46.0	96.6	(Filho et al., 1993)
	XynB	Oat spelt	7.0	492.9	
	XynC	Beechwood Oat spelt Birchwood	20.18 7.7 25.13	157.77 169.04 204.57	(Ryan et al., 2003)
Penicillium chrysogenum	Xylanase	Oat spelt Birchwood	4.1 4.2	1058 934	(Haas et al., 1992)
Penicillium funiculosum	XYNC	Birchwood	4.7	2,540	(Furniss et al., 2002)
Penicillium glabrum	Xylanase	Oat spelt Birchwood Beechwood	1.2 5.3 3.1	393.17 212.10 194.21	(Knob et al., 2013)
Penicillium herquei	Xylanase	Oat spelt	4.5	84.4	(Funaguma et al., 1991)
Penicillium oxalicum	Xyn11A	Beechwood Birchwood Oat Spelt	3.0 2.8 7.9	244.8 115.2 500.1	(Liao et al., 2015)
Penicillium purpurogenum	XynA	Birchwood	-	966	(Belancic et al., 1995)
	XynB	Birchwood	-	199	
Penicillium sclerotiorum	Xylanase I	Oat spelt Birchwood	2.6 6.5	241.25 189.70	(Knob and Carmona, 2010)
	Xylanase II	Oat spelt Birchwood	23.45 26.61	123.68 189.70	
Penicillium sp. 40	XynA	Oat spelt	8.3	6,100	(Kimura et al., 2000)
Penicillium verruculosum	XYN	Birchwood	-	14.7	(Berlin et al., 2000)

[a]Enzyme concentration was calculated by BCA method

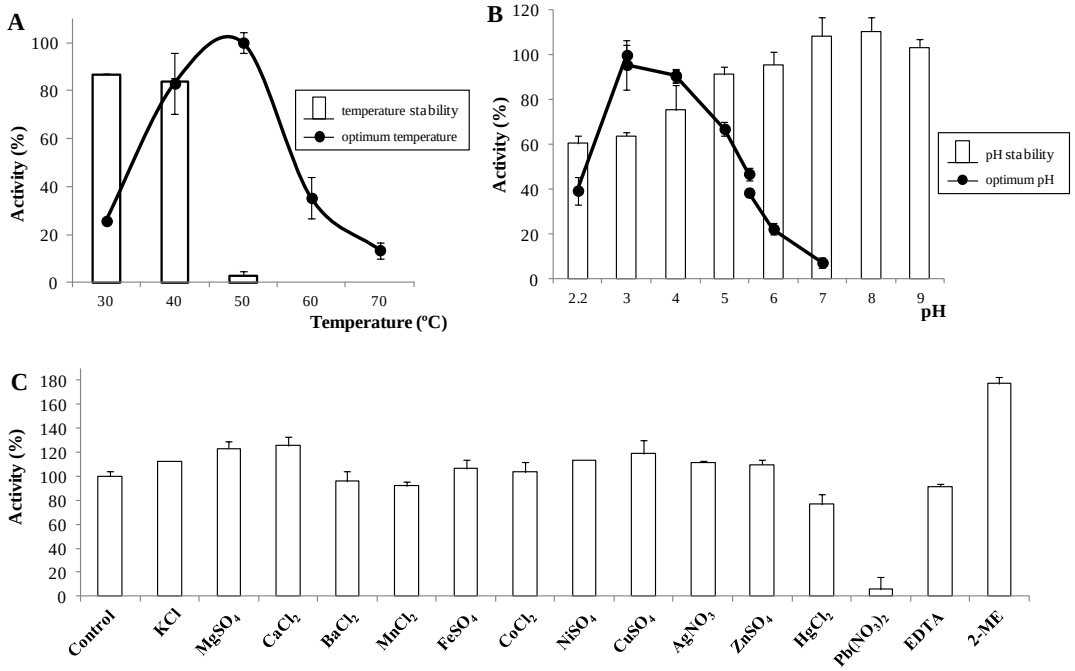


Fig. 4.24. Effect on XynM activity of: (A) pH, (B) temperature, and (C) common chemical compounds. A) The line indicates the effect of temperature on enzyme activity, and the bars show its stability over a range of temperatures from 30 °C to 50 °C after 72 h. B) The line indicates the effect of pH on enzyme activity, and the bars show its stability over a range of pH values from 2.2 to 9 after 72 h.

4.4.5. Sequencing and classification of XynM

The preliminary identification of XynM relied on its peptide mass fingerprint and N-terminal sequence. MALDI–TOF and TOF/TOF data from the fingerprint and N-terminal sequences were used to interrogate the NCBI non-redundant protein database. The search returned as the best matches a GH11 protein from *Thielavia terrestris* NRRL 8126 (gi:367042760) and Xylanase B from *Talaromyces purpurogenum* (gi:1004289) (Belancic et al., 1995) respectively.

The sequences from the mRNA encoding for *T. purpurogenum* (GenBank accession number Z50050.1) and *T. terrestris* (NCBI Reference Sequence XM_003651712.1) xylanases were used as queries to run local BLASTN against the assembled genome of *T. amestolkiae*. Both queries matched the same genome region with E values of $2 \cdot 10^{-18}$ and $4 \cdot 10^{-23}$ respectively. The located region was validated by identifying the reported internal peptide and N-terminal sequence. Then, alignment of this region with the gene sequences of XynB and the GH11 xylanase from *T. terrestris* allowed predicting the start and stop codons and the presence of a single intron.

The putative sequence of XynM, analyzed using the SignalP 4.1 server, indicated a signal peptide that fitted perfectly with the beginning of the N-terminal sequence determined experimentally. The mature gene sequence without the signal peptide and the intron was analyzed by the

ExPASY Bioinformatics Resource Portal and resulted in a theoretical molecular mass of 19,785 Da, very close to the value obtained by MALDI-TOF for the purified protein. This good agreement and the matches of the N-terminal sequence and the internal peptide suggest that it was correctly identified. The nucleotide sequence was then submitted to the GenBank database under the accession number KX641268.

The predicted gene was analyzed by the dbCAN server in order to annotate the enzyme into a glycosyl hydrolase family, indicating that XynM belongs to the GH11 family (E-value $4.7 \cdot 10^{-58}$). This was not unexpected attending to its low molecular mass and high specificity reported above (Polizeli et al., 2005).

4.4.6. Production of XOS by hydrolysis of birchwood xylan catalyzed by XynM

XOS production reached maximum yield after 10 h reaction, and the profile of DPs barely changed for the following 14 h (Fig. 4.25A, Table 4.12). The thermal and end-product inhibition of XynM as well as the decrease of accessible hydrolytic sites in the polysaccharide may explain the stagnation of the global yield (Akpinar et al., 2010). The maximum output of neutral XOS (28.8%) is expressed as percentage of the initial amount of birchwood xylan, and was calculated from the sum of the concentrations of uncharged XOS from X2 to X6 measured from HPAEC-PAD (Table 4.12). The high content of X2 and X3 in the reaction mixture using XynM agrees with the fact that GH11 endoxylanases do not display significant activity towards these small products (Pollet et al., 2010). It is noteworthy the negligible formation of free xylose. Other reaction products could not be identified, and probably comprise substituted xylooligosaccharides. Indeed, the high retention times observed for the unidentified XOS suggested that these substituents may be negatively charged, which would be in good agreement with the presence of glucuronic or methyl-glucuronic acids, some of the most common side-chains in xylans. In fact, this is the case of birchwood xylan, which has been highlighted for containing considerable amounts of glucuronic acid (Kormelink and Voragen, 1993).

Mass spectrometry analysis confirmed the presence of 4-*O*-methyl-D-glucuronic acid derivatives in the reaction. The peaks were found as $[M + Na]^+$ and $[M + K]^+$ adducts in the positive mode (Fig. 4.25B) and as $[M – H]^-$ and $[M+Cl]^-$ adducts in the negative mode (Fig. 4.25C). Sodium and deprotonated adducts were found to be more stable when consider xylooligosaccharides with higher degree of polymerization, an observation which has been previously reported in the case of sodium adducts (Aachary and Prapulla, 2009). The presence of xylose (173.04 m/z), glucose (203.05 m/z), xylobiose (305.08 m/z; 321.05 m/z), xylotriose (437.20 m/z; 453,09

m/z) and xylotetraose (569.16 m/z) was confirmed, in agreement with the previous results obtained by HPAEC-PAD (Fig. 4.25A). In the negative mode spectra, 4-*O*-methyl-α-D-glucuronopyranosyl derivatives of xylooligosaccharides X3-X5 (603.18 m/z; 735.22 m/z; 867.26 m/z) were also found. The absence of larger neutral XOS may seem surprising since they were identified in the HPAEC-PAD analyses. However, their low proportion in the reaction mixture as well as the reduced ionizability of this type of oligosaccharides (Ahn and Yoo, 2001) can prevent the detection by ESI-MS.

Table 4.12. Composition of the reaction mixture in the hydrolysis of 2% (w/v) birchwood xylan with XynM. Glc: glucose; X1: xylose; X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5: xylopentaose; X6: xylohexaose; GA: glucuronic acid; XOS: xylooligosaccharides.

Reaction time	**[Products] (g/L)[a]**								**XOS yield**
(h)	**Glc**	**X1**	**X2**	**X3**	**X4**	**X5**	**X6**	**Total[b]**	**(%)[c]**
0.25	0.05	0.01	0.50	0.93	0.76	0.23	0.43	2.90	14.2
1	0.11	0.02	1.03	1.55	1.11	0.27	0.40	4.50	21.80
3	0.12	0.03	1.18	1.79	1.26	0.31	0.47	5.16	25.0
5	0.11	0.04	1.21	1.70	1.19	0.29	0.45	4.98	24.1
7	0.12	0.05	1.33	1.85	1.29	0.31	0.49	5.45	26.4
8	0.13	0.06	1.39	1.90	1.32	0.32	0.50	5.61	27.1
10	0.13	0.06	1.49	2.01	1.39	0.33	0.53	5.95	28.8
24	0.14	0.08	1.53	1.78	1.24	0.31	0.57	5.65	27.2

[a] Determined by HPAEC-PAD.
[b] Total identified products.
[c] Percentage of X2-X6 referred to the initial amount of birchwood xylan.

The use of endoxylanases for obtaining XOS from xylan as an alternative to the physicochemical approaches has attracted great interest and several reports have come out during the last decade (Table 4.13). It should be emphasized that comparison of the published information on different xylanases is a difficult task, since parameters as enzyme dosage and purity (especially regarding the presence or absence of xylanolytic auxiliary activities), xylan source, and type of pretreatment vary depending on the report.

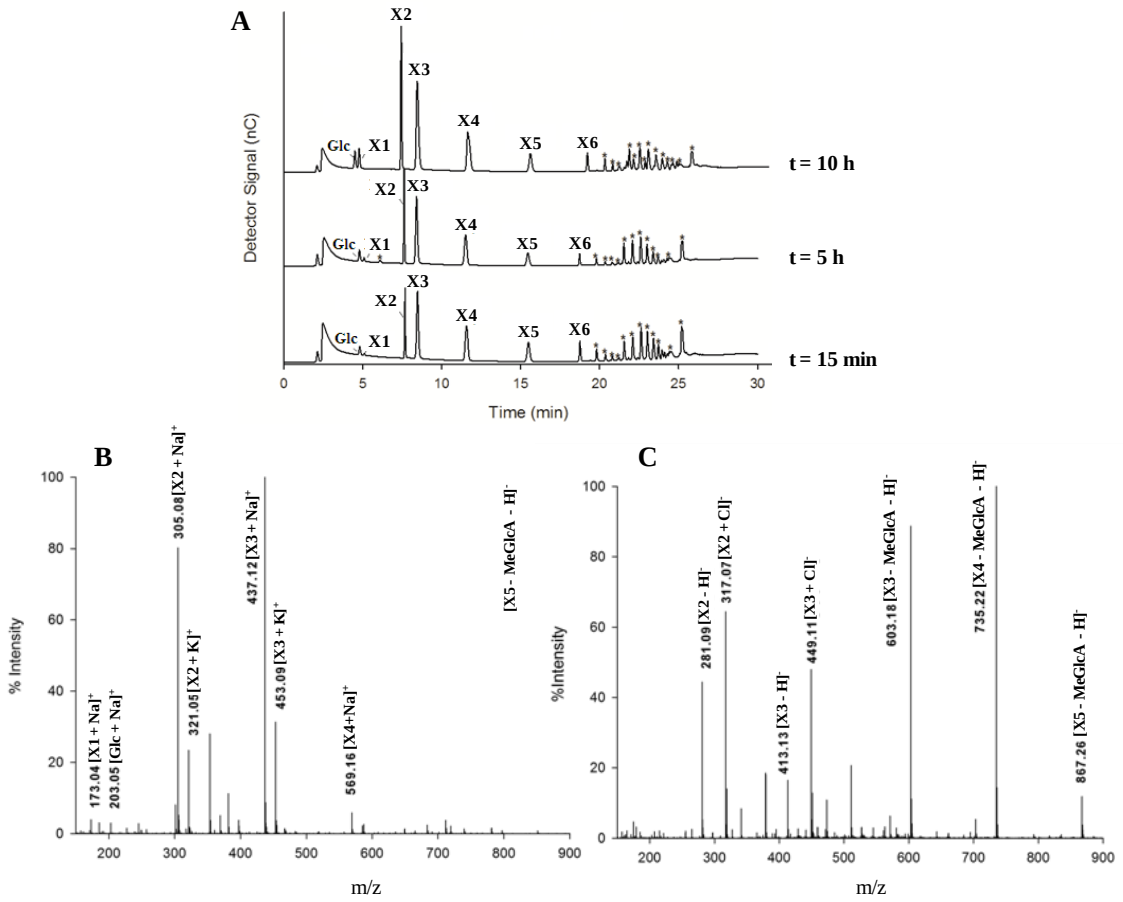


Fig. 4.25. Hydrolysis of 2% (w/v) birchwood xylan with XynM. A) HPAEC-PAD chromatograms from 15 min, 5 h and 72 h-reaction mixtures. B) ESI/MS spectra recorded in the positive ion mode; C) ESI/MS spectra recorded in the negative ion mode. Glc: glucose; X1: xylose; X2: xylobiose X3: xylotriose; X4: xylotetraose; X5: xylopentaose; X6: xylohexaose; MeGlcA: 4-*O*-methyl-D-glucuronic acid; *: unidentified.

However, the analysis of data from the literature revealed that XynM produced XOS in a comparable yield to those from most of the reported xylanases (Akpinar et al., 2007; Bian et al., 2013; Teng et al., 2010) releasing a virtually negligible amount of monomeric xylose, which is indeed the main advantage of the enzymatic production of XOS in comparison with physicochemical approaches.

Table 4.13. XOS production yields from different enzymatic hydrolysis and xylan sources.

Enzyme source	Xylan source	Enzyme dosage (U/g)	[XOS] (%)	[Xylose] (%)	Reference
Fungal xylanases					
T. amestolkiae	Beechwood	50	28.8	0.06	This work
Pichia stipitis	Sugarcane bagasse	25	31.8	0.27[a]	(Bian et al., 2013)
Aspergillus foetidus	Corncob	Not reported	60.0	Absence	(Chapla et al., 2012)
Trichoderma viridae	Sugarcane bagasse	Not reported	8.1[a]	4.0[a]	(Jayapal et al., 2013)
Pichia stipitis	*Populas tormentosa*	25	36.8	0.2	(Yang et al., 2011)
Aspergillus niger	Tobacco stalk	20.5[a]	11.4	0.7	(Akpinar et al., 2010)
Thermoascus aurantiacus	Sugarcane bagasse	60	37.1	24.1	(Brienzo et al., 2010)
Paecilomyces thermophila	Corncob	210[a]	28.6	7.7[a]	(Teng et al., 2010)
Aspergillus oryzae	Corncob	200[a]	86.7	13.3	(Aachary and Prapulla, 2009)
Bacterial xylanases					
Bacillus subtilis	Sugarcane bagasse	40	>99	0.4	(Reddy and Krishnan, 2016)
Bacillus halodurans	Wheat straw	96[a]	36	2.4	(Faryar et al., 2015)
Bacillus mojavensis	Garlic straw	12	29	Not reported	(Kallel et al., 2015)
Rhodothermus marinus	Wheat bran	63	59.0	Not reported	(Immerzeel et al., 2014)
Rhodothermus marinus	Birchwood	25.6[a]	20	0.2	(Falck et al., 2013)
	Rye flour	33.4[a]	3.3	0.1	
Bacillus subtillis	Wheat bran	25	6.4	Not reported	(Wang and Lu, 2013)
Thermobifida fusca	Oat-spelt	500[a]	40.1	0.61	(Yang et al., 2007)
	Corncob		29.5	0.61	
	Bagasse		23.7	0.57	
	Wheat bran		7.6	0.39	
	Peanut shell		10.1	0.85	

[a] Not included in the original article but calculated with data provided.

4.4.7. Prebiotic effect of XOS from birchwood xylan

Production of SCFAs and other organic acids.

SCFAs, lactate and succinate, some of the main end-products of fermentation by colonic bacteria (Fooks et al., 1999; Rodriguez-Colinas et al., 2013), were determined in fecal fermentations supplemented with the whole mixture of birchwood XOS (containing both the neutral XOS and the 4-*O*-methyl-D-glucuronic acid derivatives) produced by catalysis with XynM. The global production of organic acids was remarkably higher in the presence of these XOS, especially for acetic and, to a lesser extent, for lactic acid, and the differences observed seemed to be dose-dependent (Fig. 4.26). In general, this profile is in good agreement with other reports for fecal fermentations of XOS (Kabel et al., 2002). However, the amount of butyric acid, an end-product usually found upon fermentation of other mixtures of XOS by probiotic bacteria (Lecerf et al., 2012), changed from 14 mg/L (0 h) to be negligible regardless of the presence or not of the XOS mixture.

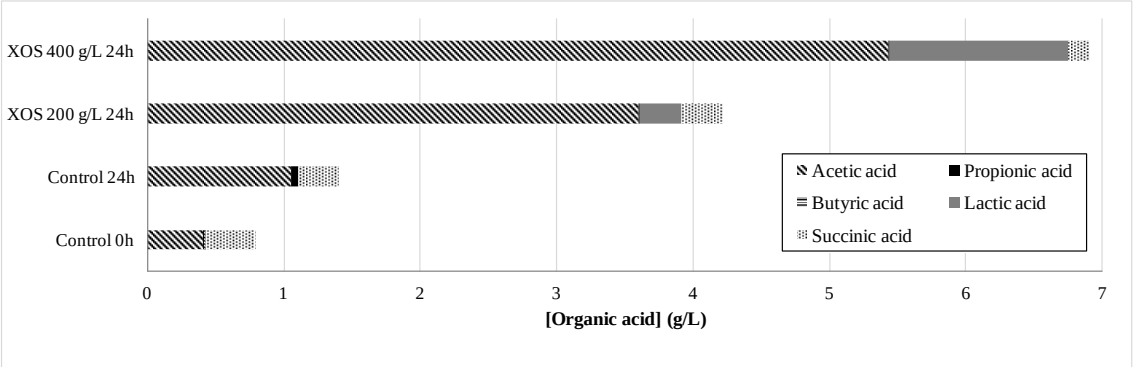


Fig. 4.26. Profile of SCFAs, lactic and succinic acid in the fermentations with XOS and controls.

Even though the presence of these organic acids, which are well-known biomarkers for probiotic species (Date et al., 2014; Gullon et al., 2011), suggested the prebiotic effect of birchwood XOS, its role was further confirmed analyzing the composition of the microbial communities in these samples.

Microbiome determination.

Microorganisms were identified up to the genera and putative species level and quantified in terms of relative abundance (Fig. 4.27). According to the microbiome data, Bifidobacterium was one of the predominant genera in the initial control (25%). The abundance of these probiotic bacteria in the feces of breast-fed children has been previously reported and related to the presence of prebiotic oligosaccharides in breast-milk (Barile and Rastall, 2013). However, the proportion of Bifidobacterium species

dropped off remarkably (18%) after 24 h in the control without XOS, while a clear beneficial effect of the XOS mixture on bifidobacteria was observed. The presence of 200 g/L XOS allowed maintaining its initial population (23%) in the same time period, and with 400 g/L it raised up to 32%.

In the case of *Lactobacillus,* the other fully established probiotic genus, the presence of the tested XOS seemed to have no impact in its relative abundance, as it has been noticed for other XOS mixtures (Li et al., 2015b).

Apart from the effect on conventional probiotic bacteria, the most remarkable observation was the dramatic increase of the proportion of *Staphylococcus* sp., and specifically *S. hominis* in the presence of XOS respect to the control samples at 0 h and 24 h. Given the extent of the increase, the identity of this species was further confirmed by PCR-based analysis (Table 4.14).

Table 4.14. Results of BLASTN search for the identification of *S. hominis* from specific PCR-based assays.

Gene	Query cover	Identity	Score	E-value	Species	GenBank ID
rpoB	100%	98%	990	0.0	*S. hominis*	LM651927.1
tuf	100%	100%	542	$2 \cdot 10^{-150}$	*S. hominis*	HM071882.1

S. hominis is a coagulase-negative staphylococcus naturally found in the human microbiome of breast-fed children (Martin et al., 2007). Strains of this species were recently suggested as potential probiotics for its ability to produce bacteriocins, a sort of substances that act as growth inhibitors of relevant pathogens as *Staphylococcus aureus* or *Helicobacter pylori* (Lopez-Brea et al., 2008; Sung et al., 2010). The enrichment in *S. hominis* may be related to the observed profile of organic acids. The high quantities of acetic and lactic acids determined in samples containing XOS may seem surprising when compared to the modest increase in the abundance of bifidobacteria. As observed in Figure 4.26, the content of acetic acid increased 3.6- and 5.4-fold in fermentations with 200 g/L and 400 g/L XOS samples, respectively, as compared with the 24 h control. Indeed, the production of these metabolites is related to the two main bacterial populations positively affected by XOS: *Bifidobacterium* (~1.8-fold increment) and *S. hominis* (12.0-fold increment with 200 g/L XOS and 14.7-fold with 400 g/L). Considering the evident stimulation of the growth of the last microorganism by XOS, and taking into account that it was found to produce both acetic and lactic acid (Julak et al., 2000; Kloos and Schleifer, 1975), the organic acids profile determined in these experiments could be mostly attributed to *S. hominis* metabolism. However, it is worth mentioning that, besides the role of *S. hominis,* there are other potential

explanations for the acids' profile, as this pattern can differ for the same bacterial population depending on the available substrates.

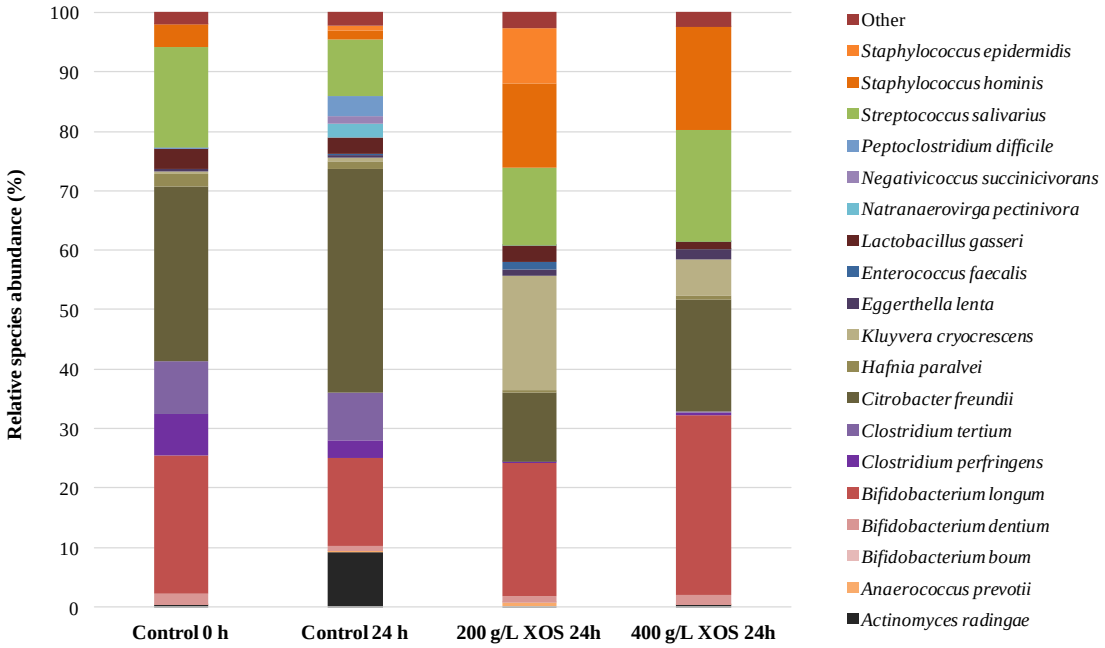


Fig. 4.27. Relative species abundance of bacterial microbiomes from fermentations of control fecal samples (0 and 24 h) and in 24 h-samples containing a mixture of XOS.

To a minor extent, results regarding *Streptococcus salivarius* are also interesting. This specie was also reported as a potential probiotic (Burton et al., 2006) and its content increased respect to the 24 h control from 11% to 19% in samples with 400 g/L XOS.

The presence of two clostridia in control samples is also notable (16% at 0 h and 12% after 24 h). One of them, *Clostridium perfringens*, is a normal member of the gut microbiota that has been related to several diseases when its population increases to pathogenic levels (Smedley et al., 2005). The data in Figure 4.27 show that the presence of birchwood XOS led to a decrease in the abundance of clostridia below 0.5% for both concentrations tested. XOS also prevented the development of other pathogens, as *Actinomyces radingae* (7% microbiome of the 24 h control sample) that can produce actinomycosis, a human chronic disease (Smego and Foglia, 1998). Enterobacteriaceae was the most represented family in the control microbiomes, constituting 32% and 37% at 0 h and 24 h respectively. Among the members of this family, *Citrobacter freundii* was a clearly dominant species in controls, while its representation in the microbial community decreased notably. Just the opposite effect was observed for *Kluyvera cryocrescens*, which displayed a remarkable growth with 400 g/L XOS and was the main enterobacteria with 200 g/L. The biological relevance of this change in the microbiota profile is not clear and more information about both species is required. In global terms, 200 g/L

XOS avoided the increase of enterobacteria while 400 g/L dropped off its presence to 26%. This decrease is considered a characteristic feature of prebiotics, which prevent the development of higher concentrations of potentially pathogenic species from this family (Macfarlane et al., 2006).

Xylooligosaccharides are considered novel candidate prebiotics (Rastall and Gibson, 2015). Together with this work, rising evidences support their role in selectively affecting gut microbiota, in particular though stimulating the growth of several species from *Bifidobacterium* and *Lactobacillus* (Aachary and Prapulla, 2011). However, most of these studies were carried out *in vitro* using single-species cultures (Chapla et al., 2012; Immerzeel et al., 2014) while the number of *in vivo* reports is much more limited and restricted to the effect of commercial XOS mixtures on adult population (Chung et al., 2007; Lecerf et al., 2012). Fermentation of fecal slurries constitute an *in vitro* alternative to pure cultures that provides a useful representation of the diversity of the gut microbiota without requiring large quantities of the prebiotic tested (Gibson et al., 2004). However, the investigations based on the effect of XOS in fecal fermentations are scarce (Gullon et al., 2011; Kabel et al., 2002).

Most studies conducted on infants focus on formulations of galactooligosaccharides (GOS) and fructooligosaccharides (FOS) that mimic the composition of human milk oligosaccharides (HMOS) and the prebiotic effect of human milk, without paying attention to other potential prebiotics (Thomas and Greer, 2010; Barile and Rastall, 2013). The main goal for those works was to restore the predominance of gut bifidobacteria, present in children fed with human breast milk, but lost in formula-fed infants (Thomas and Greer, 2010). Using this model, the XOS mixture obtained in this work has demonstrated not only bifidogenic effect, but also a certain antimicrobial action, which is typical from HMOS. Indeed, growth inhibition of species from *Clostridium* and *Citrobacter* has been previously demonstrated for human milk (Marcobal et al., 2010; Newburg and Walker, 2007). This evidence suggests the potential interest of including XOS as components of infants' formula and opens the field for determining the effect of other prebiotics beyond GOS and FOS.

5. GENERAL DISCUSSION

5.1. XYLAN: APPLICATIONS COMPLEMENTARY TO BIOFUELS

The properties and potential applications of two enzymes from the xylanolytic machinery of *T. amestolkiae*, an endoxylanase and a β-xylosidase, have been presented throughout the last four chapters. These enzymes began to be studied in-depth in the 80's because of their interest in the field of saccharification of pretreated biomass for subsequent fermentation (Linko et al., 1984) and on the metabolism of organisms capable of directly bioconverting lignocellulosic material into ethanol (Deshpande et al., 1986; Gong et al., 1981). This is still one of the main applications of hemicellulases, which are currently combined with pretreatments of widespread use as dilute sulfuric acid, in order to perform saccharification of biomass, achieving yields over 90% (Lloyd and Wyman, 2005). However, over time, the use of these biocatalysts has exceeded the field of bioenergy, extending to other fields of biotechnological relevance. The most general and commercially exploited applications are based on xylan hydrolysis as a method to improve a certain industrial process. Some remarkable examples are retting of textile fibers, juice clarification, increasing digestibility of animal feed or bleaching cellulose pulp (Collins et al., 2005; Polizeli et al., 2005). In all these cases xylan is an undesired element to remove, but there are other possibilities (also apart from ethanol production), less developed at the moment, that use this polysaccharide as raw material to obtain added-value products.

Regarding to its main field of application, in order to understand the role of xylanases in the production of 2G ethanol, it is necessary to make a brief summary about the origin and development of biofuels. They emerged as an alternative to fossil fuels, which are limited resources whose use is largely responsible for climate change. Focusing on bioethanol, its development started with the so-called first generation technology, which requires agricultural products with high sugar content, as beet, sugarcane, or cereal grain. This technology, strongly encouraged by public policies at the beginning of this century, expanded the world production of bioethanol between the years 2000 and 2010 (from $1{,}7 \cdot 10^{10}$ L to $8{,}8 \cdot 10^{10}$ L), whereas in the previous decade the increase was much more limited (~13%) (Guo et al., 2015). Nevertheless, the food crisis of 2007-2008, raised during full expansion of the “energy crops”, forced governments to rethink the use of food products for the obtaining of biofuels (Escobar et al., 2009; McMichael, 2009). The interest in 2G bioethanol emerged in this context, because it uses lignocellulosic biomass as raw material, thereby not detracting resources potentially destined for food.

Unfortunately, 2G bioethanol involves its own drawbacks, since the hydrolysis of plant cell wall polysaccharides is more difficult and requires the previous disruption of the lignin barrier, so the cost of the global

process increases largely in comparison to 1G ethanol production. In recent years, considerable efforts have been done in order to reduce this cost, among those, the exploitation of hemicelluloses in addition to cellulose, together with the search of yeasts capable of fermenting both xylose and glucose are especially remarkable (Gírio et al., 2010; Ko et al., 2016). However, nowadays, the yields are still low and the commercial viability of the process requires obtaining other value-added products from all the components of lignocellulosic biomass. This integrative approach receives the name of biorefinery (Ragauskas et al., 2006). In this sense, the sugar nature of hemicelluloses, and especially xylans because of their abundance, would involve, *a priori*, their conversion into ethanol. Nevertheless, both xylan saccharification and further fermentation display certain drawbacks.

As mentioned above, biomass saccharification requires a previous pretreatment for removing the lignin barrier allowing the access to polysaccharides. There are several types of pretreatments extensively used and each of them affects the hemicellulosic component differently (Table 5.1).

Table 5.1. Some advantages and disadvantages of the most common pre-treatments. Adapted from Gírio *et al*. (2010).

	Pre-treatment				
Desirable features	Concentrated acid	Dilute acid	Steam explosion	Autohydrolysis	Alkaline
High xylan solubilisation	++	++	++	++	+
High xylose production	++	++	0	−	−/0
Low XOS production	+	+	0	−	−/0
Low inhibitors formation	−	−	0	0	+
Low energy use	0	−	0	0	+

+, Advantage; −, disadvantage; 0, neutral.

Thus, procedures using concentrated or dilute acid (H_2SO_4) achieve the high solubilisation of xylan, simultaneously with its conversion into xylose. Unfortunately, large quantities of inhibitors are generated in these conditions, especially phenolic compounds, derived from lignin, and furfurals, produced by degradation of pentoses. By this way the yield of the process diminishes, not only due to the loss of fermentable sugars, but also because these by-products are toxic for the microorganism carrying out fermentation (Hahn-Hägerdal et al., 2006; Jurado et al., 2009). Other approaches, as steam explosion or autohydrolysis, decrease the formation

of inhibitors, but require higher levels of xylanolytic activities in the enzymatic cocktails for saccharification in order to complete the hydrolysis of xylan (Gírio et al., 2010; Kumar and Wyman, 2009). Finally, in regard to the fermentation process, the most used yeast is *Saccharomyces cerevisiae*, which is not able to convert xylose into ethanol. This circumstance has forced to search for alternative fermenting species or, in recent years, to develop *S. cerevisiae* strains carrying genes of the pathway for xylose utilization from other yeasts (Cadete et al., 2016; Nogue and Karhumaa, 2015).

Alternatively, during this decade, many studies have been reported suggesting other possibilities for the exploitation and valorization of xylan, apart from its conversion into bioethanol. For these applications hemicellulose can come from any of the pretreatments for biofuels production or from other industrial sectors, being particularly relevant the pulp and paper industry. Among the proposed uses, it can be found: 1) production of biomaterials, as biodegradable coatings or polymers for the controlled release of drugs (Chaa et al., 2008; Oliveira et al., 2010; Ruiz et al., 2013); 2) the obtaining of antioxidants as ferulic acid glycosides (Ebringerova et al., 2008; Yuan et al., 2005) or 3) its conversion into xylitol, one of the most promising emerging sweeteners (Guo et al., 2013). In addition to these options, other applications have been described along the previous chapters, both for xylan and xylanolytic enzymes, which will be discussed in the sections below.

5.2. *Talaromyces amestolkiae*: A SOURCE OF XYLANOLYTIC ENZYMES

The promising features of the ascomycete *T. amestolkiae* as producer of cellulolytic enzymes (Gil-Muñoz, 2015), together with preliminary tests suggesting that it may also be a source of xylanolytic enzymes, determined the planning of this doctoral thesis. Thus, it was decided to perform a global evaluation of the fungal potential in producing hemicellulases for plant biomass valorization. The work focused on the production and characterization of endoxylanase and β-xylosidase activities, because of their central roles in the degradation of xylan, which is the main component of the hemicellulose fraction in wheat residues, a very abundant cereal in Spain. The production of these enzymes was initially tackled by culturing the fungus in a basal medium, in the presence of beechwood xylan or microcrystalline cellulose as inducers or glucose as carbon source (Chapters I and IV).

Xylan has been widely characterized as a good inducer of hemicellulases in many lignocellulolytic organisms, and specifically in

fungi from the genus *Penicillium* (Chavez et al., 2006), hence the secretion of high levels of hemicellulolytic activity by *T. amestolkiae* may be expected in the presence of this heteropolysaccharide. In addition, the use of commercial xylan, highly pure, generally leads to results displaying good reproducibility and easily comparable to those reported for other organisms. Regarding to microcrystalline cellulose, this substrate, a strong inducer of cellulolytic activities, was included in the study due to the close interrelation between the metabolisms of cellulose and hemicelluloses reported for many filamentous fungi (Aro et al., 2005). In this sense, it has been demonstrated that *T. reesei*, in the presence of cellulose, sophorose or β-glucans as sole carbon sources, actives the expression of not only endoxylanases and β-xylosidases, but also auxiliary enzymes as acetylxylan esterases and α-L-arabinofuranosidases. In the same way, the use of xylan induces the production of hemicellulases and also cellulases (MargollesClark et al., 1997). One explanation for this behavior is related to the occurrence of the transcriptional activator XlnR. This factor controls the expression of several cellulases and hemicellulases, both exo- and endo-type, in *A. niger* and homologous activators have been identified in other species of the genera *Aspergillus*, *Trichoderma* and *Penicillium* (Aro et al., 2005). Finally, the production of endoxylanases and β-xylosidases was evaluated in the presence of glucose, since this monosaccharide is a well-established inhibitor of the xylanolytic metabolism in the genus *Penicillium* (Chavez et al., 2006).

The results obtained from this study were in good agreement with those expected for the different carbon sources selected. Thus, maximal level for endoxylanase and β-xylosidase activities were detected by using 2% (w/v) beechwood xylan, and this concentration was kept in the production of XynM and BxTW1 by *T. amestolkiae* for their further purification and characterization. In the presence of cellulose the noticed activities were much lower in comparison to the values settled for xylan, which may be attributed to the mentioned overlapping between metabolisms or to the constitutive expression reported for some xylanolytic enzymes. Finally, the effect of glucose was the expected inhibition of both analyzed activities. Despite *T. amestolkiae* produced these two enzymes at levels better than those reported for many *Penicillium* species (Ben Romdhane et al., 2010; Knob et al., 2013; Liao et al., 2012; Terrasan et al., 2010), their maximal values were similar or lower than those secreted by some ascomycetes belonging to other genera (Haltrich et al., 1996; Lenartovicz et al., 2003).

The analyses performed on induction of the xylanolytic metabolism in *T. amestolkiae* were later validated by de Eugenio *et al.* (2017) that studied the secretomes produced by this microorganism in the presence of each of the carbon sources cited above or a complex lignocellulosic

substrate: wheat straw pretreated by steam explosion with acid. The results of the report were, in a large extent, those expected on the basis of the production curves presented in Chapters I and IV of this thesis (Figs. 4.2 and 4.22). Thus, the maximal levels of xylanolytic enzymes were obtained in the medium with beechwood xylan and these biocatalysts constituted about 16% of the total secreted proteins. This value decreased remarkably in the presence of microcrystalline cellulose (~6%), whereas with pretreated wheat straw an intermediate result was observed (~10%). Cultures with glucose displayed the foreseeable absence of endoxylanases and β-xylosidases. Further analysis of the secretome released in the presence of these carbon sources revealed that the fungus produced another endoxylanase, in addition to the GH11 protein already characterized (Chapter IV), which was classified as a GH10 glycosidase. Regarding the β-xylosidase activity, BxTW1 (GH3) was detected together with three novel bifunctional enzymes, also annotated as α-L-arabinofuranosidases, two belonging to family GH3 and one to family GH43. Among the rest of secreted proteins a wide variety of auxiliary xylanolytic enzymes were found, confirming that this ascomycete produces, at least, one acetylxylan esterase (CE2), one feruloyl esterase (CE1) and a surprising number of α-L-arabinofuranosidases, including the three bifunctional enzymes mentioned above and seven more, distributed among families 43, 51, 54 and 62. The presence and levels of each one of these CAZymes display an apparent pattern of differential expression depending on the selected carbon source. It can be highlighted that pretreated wheat straw induces the highest number of different xylanolytic enzymes, probably because it is a complex lignocellulosic substrate which requires a high number of activities for being degraded.

The results from de Eugenio *et al*. (2017), also represent an additional support for some of the hypothesis stated in Chapters I and IV. Thus, the detection of other xylanolytic activities different from endoxylanase in the cultures induced by beechwood xylan confirms the loss of relevant CAZymes for the hydrolysis of this polysaccharide during the purification of XynM. By this way, as previously proposed, the apparent low yield associated to the isolation of the enzyme may be explained. A more detailed qualitative analysis of the fungal secretomes revealed a remarkable feature: in spite of the inferior xylanolytic levels induced by microcrystalline cellulose, this secretome contained a higher number of different enzymes and activities involved in the hydrolysis of xylan than the cultures induced with this hemicellulose itself. This notable abundance of CAZymes might indicate that the response of this organism to cellulose is subjected to a wider and more complex regulation than the one activated by the presence of xylan. In conclusion, the reported data suggest that, in addition to BxTW1 and XynM, further analysis may lead to

the isolation of novel hemicellulases with biotechnological interest. In this sense, the diversity of the secretome produced in the presence of pretreated wheat straw should be noted, which indicates that this type of low cost lignocellulosic materials might be good inducers, replacing commercial xylans or microcrystalline cellulose that are less interesting from an industrial perspective.

5.3. XynM: PRODUCTION OF XYLOOLIGOSACCHARIDES, EMERGING PREBIOTICS

Endoxylanases are one of the most studied enzymes in the field of glycosidases active on biomass, only preceded by cellulases. The first works on the detection of this activity (Iwata, 1935) and the production of xylanolytic preparations (Inaoka and Soda, 1956; Sørensen, 1952) were reported more than fifty years ago and the tridimensional structures associated to families GH10 and GH11 (at that time called F and G, respectively) are known since the 90s. In the same way, neither their importance in the production of bioethanol (Lee et al., 1986) nor in the paper industry (Senior et al., 1989) are recent discoveries. On the contrary, the use of these enzymes for production of xylooligosaccharides because of the prebiotic properties associated to these carbohydrates constitutes a novel and emerging field (Aachary and Prapulla, 2011; Linares-Pastén et al., 2017), opening new expectatives for the application of endoxylanases.

In this doctoral thesis the preparation of XOS was accomplished using XynM, an endoxylanase from *T. amestolkiae*. It was purified from fungal supernatants containing beechwood xylan as carbon source to induce the xylanolytic activities. The purification yield was quite low, although the activity loss was probably due to the unavoidable removal of other xylanolytic enzymes along the two chromatographic steps required for XynM isolation rather than to the own process. The purified endoxylanase displayed properties typical from family GH11 and this classification was confirmed by the sequencing of its coding gene.

The hydrolysis of birchwood xylan catalyzed by XynM showed a yield similar to the ones reported for other fungal and bacterial xylanases. Regarding the product profile, it included the expected mixture of neutral and charged xylooligosaccharides, attending to the nature of xylan substituents. Their quantification, restricted to the compounds with available commercial standards, revealed the predominance of xylobiose, xylotriose and xylotetraose. The monosaccharide was found just in residual traces, which is considered the main advantage of enzymatic approaches over physicochemical hydrolysis. Once studied this production profile, the process was performed at larger scale and the prebiotic properties of the

complete mixture of XOS was assayed in fecal fermentations. This is an intermediate procedure between pure cultures, excessively simple, and *in vivo* assays, which require large quantities of the tested prebiotic. One of the main innovations of the study was the utilization of feces from a breast-fed baby, while in previous reports the fecal material was from adult individuals. The potential prebiotic effect of XOS was determined by analyzing the profile of organic acids, a frequent approach for fecal fermentations, together with the study of the microbiomes developed in each condition. Bacterial populations were evaluated in order to define genera and species, and quantifying both categories in terms of relative abundance. Notably, although this type of determinations have been carried out previously, they were always associated to studies using commercial mixtures of XOS (Childs et al., 2014), therefore obtained by a physicochemical approach, which, generally, involves a process of purification after production (Qing et al., 2013). The results from the fecal fermentation performed in the present study also revealed novel data, as the selective growth promotion of *Staphylococcus hominis*, a species considered potentially probiotic because it produces bacteriocins able of inhibiting pathogens. Regarding to the classical probiotics, birchwood XOS were capable of maintaining or increasing (dose-dependent effect) the initial high levels of *Bifidobacterium* species that are associated with breast-fed children. Therefore, the data obtained suggest that the field of prebiotic formulations for infants, currently restricted to fructo- and galactooligosaccharides, might be open in the future to other types of prebiotics as xylooligosaccharides.

5.4. BxTW1: IDENTIFICATION OF A PROMISING β-XYLOSIDASE FOR TRANSXILOSYLATION

Analysis of the secretome of *T. amestolkiae* cultured with beechwood xylan revealed the presence of four CAZymes annotated as β-xylosidases, or bifunctional β-xylosidases/α-L-arabinofuranosidase (de Eugenio et al., 2017). However, despite this diversity, BxTW1 showed to be the only enzyme with this activity isolated from the fungal crudes in those conditions (Chapter I). This can be a consequence of its higher abundance in comparison to the rest of β-xylosidases in the secretome (de Eugenio et al., 2017), but also to a better specific activity and/or stability of this glycosidase in the conditions of purification.

Once isolated, the first steps in the characterization of the enzyme were focused on its hydrolytic potential, revealing one of the greatest catalytic efficiencies reported for a fungal β-xylosidase against *p*NPX and, more importantly, the highest against XOS from 3 to 6 units compared to

any other β-xylosidase in literature. Kinetic analysis also demonstrated the role of D-xylose as a competitive inhibitor for BxTW1 (product inhibition), which is in good agreement with its reported role for most fungal β-xylosidases (Knob et al., 2010). Biochemical assays showed other interesting properties, as its remarkable tolerance to Cu^{2+}, a metal present in many biomass residues that can decrease the saccharification yield (Bin and Hongzhang, 2010), its high stability in a wide range of pH, and its ability to use xylan as substrate. The next step in the characterization of the enzyme was the sequencing of its coding gene, which led to the inclusion of this glycosidase in family GH3. The classification of BxTW1 into this family suggested its possible application to synthetic reactions taking into account that transglycosylation has been commonly reported for other GH3 glycosidases (Bohlin et al., 2013; Eneyskaya et al., 2007; Turner et al., 2007).

As previously mentioned, the ability to transfer carbohydrates to different acceptors can be associated to many applications of biotechnological interest. However, the role of this activity in fungal physiology is still far from being understood. Regarding glycosidases used in plant biomass saccharification, it has been proposed that some β-glucosidases participate in the induction of the cellulolytic system. Some of them can catalyze the synthesis of sophorose (β-D-glucopyranose-(1→2)-D-glucopyranose) by transglycosylation from cellobiose as donor. Then, the released new disaccharide would act as a strong inducer of the expression of some cellulases (Fowler and Brown, 1992; Suto and Tomita, 2001) giving by this way a physiological role to the synthetic capacities of β-glucosidases. In the case of transxylosylation, the available information about its function in the fungal metabolism is much more limited. In this sense, the work of Badhan *et al*. (2007) is remarkable, because some bioactive xylosides were identified in the cultures of an asomycete from the genus *Myceliophthora*. According to that work, these carbohydrates are generated by transglycosylation and display an effect of induction on the fungal xylanolytic metabolism. Even though in this case transxylosylation is performed by endoxylanases, it is possible to assume that the synthetic properties of fungal β-xylosidases in other species can form these or similar xylosides, sharing the inducer function.

Regarding BxTW1, beyond its possible roles in the natural physiology of *T. amestolkiae,* most efforts have been focused on determining its potential as a biotechnological tool. The first evaluation of its transxylosylation capacities consisted on the use of simple alkan-ols as potential acceptors, which were added in large excess to the reaction mixtures. This assay aimed to analyze the formation of alkylglycosides, since the synthesis of this type of compounds is one of the most feasible transglycosylation processes, and it can be catalyzed by most of the

enzymes with synthetic abilities. In addition, the combination of an aryl-glycoside as donor and a simple alcoholic acceptor, in excess, is one of the most cited approaches in literature and it has been used for some of the first known fungal glycosidases (Takano and Miwa, 1950). By this way the capacity of BxTW1 to form alkylxylosides was demonstrated, which led to extend the search of potential acceptors to sugar alcohols, monosaccharides and disaccharides. This latter assay was successfully passed by the enzyme, which catalyzed transxylosylation reactions using the three types of acceptors. This was the first clear indicator of the biotechnological interest of BxTW1 since, although sugar alcohols and monosaccharides are still considered simple, disaccharides are more complex compounds. It should be emphasized that there are few reports on β-xylosidases able to use disaccharides as acceptors. Among them, the works of Kurakake (2005) and Dilokpimol (2011) stand out and present the quantification of the trisaccharides formed. Both β-xylosidases, produced by *Aspergillus* species, reached remarkably low yields in comparison to BxTW1 for the same disaccharide acceptors. These results not only confirm the potential of the β-xylosidase from *Talaromyces* in the synthesis of oligosaccharides, but also suggest that bioactive molecules of commercial relevance might be valid acceptors, even considering that they are generally too complex for being glycosylated by most of the known β-xylosidases.

As discussed above, the most frequent beneficial effect of carbohydrate addition is the enhanced solubility of the new glycoside as compared to that of the aglycon. In addition, this can be accompanied by improvements of stability, biosafety and even increase the biological activity of the non-glycosylated precursor (Kähkönen and Heinonen, 2003; Kim et al., 2006). In the specific case of transxylosylation, it should be considered that obtaining xylosides of food or pharmacological value display, *a priori*, a drawback: the human organism lacks of the efficient machinery for hydrolyzing β-linked xyloses. This is relevant since deglycosylation is a required step to transport many bioactive compounds to the intracellular space (Németh et al., 2003). However, there are also examples of glycosides that can be incorporated without previous disruption (Boyer et al., 2004).

Despite this circumstance, the transfer of xylose units, in comparison to the common choices as glucose or other hexoses, shows the special value of its higher reactivity, from a physicochemical perspective, which is due to the very nature of pentoses (Laroque et al., 2008; Lievonen et al., 2002). The addition of xylose has proved to be a destabilizing factor when it occurs on glycosidic derivatives of some polyphenols as anthocyanins (Sadilova et al., 2006). This singularity may confer to the xylosylated bioactive compounds distinctive features to other types of glycosylation. Given that the available information about the obtaining of xylosides is

scarce, it was decided to carry out an in-depth study of BxTW1 as a tool for transxylosylation.

Because of its importance, regioselectivity was one of the first properties analyzed. This characteristic of many enzymes is, in fact, the main advantage over chemical synthesis of glycosides, which also lacks stereoselectivity. Nevertheless, despite the fact that glycosidases display a higher specificity for a certain hydroxyl group, usually their regioselectivity is not absolute and side glycosides are generated (Kato et al., 2002; Torres et al., 2011), which complicates the purification of the desired compound and contributes to decrease the global yield of the process. Xylobiose and xylose were selected as acceptors in order to study the regioselectivity of BxTW1. High selectivity was expected for the former, since the disaccharide structure probably restricts the available positions of the acceptor in the active site. On the contrary, the presence of secondary products was probable in the case of xylose because of its small size, which may facilitate the transfer to any of its hydroxyl groups with similar physicochemical properties. Surprisingly, the analysis of both reactions by NMR demonstrated the exclusive formation of β-1,4 linkages between xylose units, suggesting a highly regioselective catalysis. Unfortunately, despite this remarkable property and the promising acceptor versatility, the purification process of BxTW1, which requires three chromatographic steps, limited seriously its commercial potential.

5.5. rBxTW1: A BIOTECHNOLOGICAL TOOL FOR OBTAINING VALUABLE XYLOSIDES

The complexity of BxTW1 purification is probably caused by the presence of a high number of proteins displaying similar pI in the fungal secretome, which is clearly shown by 2D electrophoresis (Chapter I, Fig. 4.3). This circumstance severely decreases the efficiency of the cationic exchange purification, even when a high performance column is used (Mono S, GE Healthcare). In order to overcome that limitation, the heterologous expression of the fungal β-xylosidase was carried out in *P. pastoris*, a yeast belonging to the *Ascomycota* phylum, which also comprises *T. amestolkiae*. This organism is well-known for secreting high quantities of recombinant proteins and the available strains for heterologous expression are also defective in proteases (Macauley-Patrick et al., 2005), so a simple purification process may be expected, together with absence of enzyme degradation. The predictions were confirmed and by this way a high production of rBxTW1 was obtained and its purification was completed by a single chromatographic step. Both enhancements led to a remarkable increase in the process yield, which validates the suitability of

the selected approach. In this sense, although rBxTW1 had smaller maximum velocity than the native enzyme (Chapter II), probably due to changes in the glycosylation pattern incorporated by the yeast, the yields' improvement largely compensates this disadvantage. Indeed, the availability of high quantities of enzyme and its easy isolation allowed a much more systematic evaluation of the acceptor versatility of this β-xylosidase. Thus, a wide library of potential acceptors from different chemical nature was assayed by high-throughput screening. The library generated included some compounds of the former assays, as controls, together with infrequent carbohydrates, amino acids, aryl derivatives of sugars and other aromatic molecules. The results demonstrated that the promiscuity of rBxTW1 for catalyzing transxylosylation reactions is not limited to disaccharides and simple chemicals, but includes aromatic acceptors derived from benzene, phenol or naphthalene. These compounds were, *a priori*, expected to be reluctant to transxylosylation, due to the potential steric hindrances associated to these bulky structures. However, using xylobiose as donor and 2,6-dihydroxynaphthalene as acceptor, rBxTW1 catalyzed the synthesis of the first xyloside of proven commercial interest in this thesis: 2-(6-hydroxynaphtyl) β-D-xylopyranoside. This bioconjugate has been reported as a selective antiproliferative agent and, up to now, it has been produced exclusively by chemical procedures (Jacobsson and Ellervik, 2002). The optimization of the reaction was performed by surface response methodology (specifically applying a Box-Behnken design) and led to a notable increase of the synthesis yield (8.5%), although it was still lower than the one reported for the chemical approach (28%) (Jacobsson and Ellervik, 2002). Nevertheless, the enzymatic method displays the advantages of being environmentally friendly and the use of xylobiose as donor, which is a xylan derivative and therefore a renewable resource. For this reason, rBxTW1 may be considered as an outstanding biotechnological tool for valorization of lignocellulosic biomass.

Beyond naphthalene derivatives, the acceptor screening (Chapter II, Fig. 4.14) suggested that the enzyme might be used for the synthesis of a high number of bioactive xylosides. In this sense, one of the most interesting aglycone classes was that of the plant phenolic antioxidants. These compounds are mostly extracted from plant biomass sources and usually display a wide range of beneficial pharmacological properties (Dai and Mumper, 2010; Rice-Evans et al., 1997; Scalbert et al., 2005). However, their physiological benefits are frequently impaired by a low bioavailability, a property which, among others, might be enhanced by obtaining glycosylated derivatives. Because of this, an assay was designed in order to evaluate the capacity of rBxTW1 for glycosylating a series of plant antioxidants. The results indicated that transxylosylation occurred for three of them: catechol, hydroquinone and hydroxytyrosol. Unfortunately,

in the case of catechol the quantities of glycoside obtained were low and, although the yields associated to hydroquinone were better, they were far from the highest values reported in the literature (Seo et al., 2012). On the contrary, the xyloside from hydroxytyrosol was synthesized with high yield, at unprecedented values for the enzymatic production of glycosides from this phenolic derivative as discussed previously (Chapter III). The purification of the hydroxytyrosol xyloside and its characterization by NMR revealed that, between the two positions available for sugar transfer, the recombinant β-xylosidase used exclusively the primary hydroxyl, forming 3,4-dihydroxyphenyl-ethyl-*O*-β-D-xylopyranoside. This result corroborates the regioselectivity reported for transxylosylation reactions catalyzed by the native glycosidase.

The study of the bioactive properties of the hydroxytyrosol xyloside revealed that the novel compound kept the anti-inflammatory capacity of hydroxytyrosol, but, surprisingly, increased its neuroprotective effect (Chapter III, Fig. 4.21D). Taking this into account, the value of rBxTW1 as biotechnological tool for transxylosylation reactions is considered proven. Nevertheless, a complete corroboration of the bioactivity associated to this or other xylosides of interest will require performing *in vivo* assays in animal models.

5.6. FUTURE PERSPECTIVES: COMBINATION OF XYLANOLYTIC TOOLS

In spite of its regioselectivity and wide range of acceptors, the biotechnological potential of (r)BxTW1 is limited by the use of xylobiose as transxylosylation donor. This sugar displays a renewable origin, as it is the hydrolysis product of xylans, but the high cost of the commercial pure preparations makes non-viable its use at the industrial scale. In addition, its disaccharide nature is itself a drawback that hampers obtaining good yields because the xylopyranose residue of the reducing end can never be transferred. Thus, even in ideal conditions, with a transxylosylation ratio achieving 100%, half of the xylose units contained in the donor would be unproductively released.

An interesting alternative could be to produce these xylosides from the hemicellulose itself, which can be easily extracted from lignocellulosic residues in large quantities by low-cost physico-chemical methods (Puls et al., 2006; Wang and Zhang, 2006). However, although both native and recombinant BxTW1 are active against xylan (Chapters I and II), the reaction rate is too low for the efficient use of this heteropolysaccharide as donor in transxylosylation processes. One possible way of overcoming that limitation, which has already assayed with good results in preliminary

assays, is the combination of XynM and BxTW1 in an approach of simple enzymatic cascade, which would hugely increase the yields associated to xylan as donor. This proposal is based on the endoxylanase activity, which hydrolyzes the polysaccharide and releases XOS. It may be expected that, once released, these carbohydrates are efficiently used by the β-xylosidase as donors for transxylosylation reactions (Chapter I, Fig. 4.7). Remarkably, the application of both hemicellulases from *T. amestolkiae* in tandem displays a series of additional advantages. First, as XynM belongs to family GH11, the hydrolysis would take place in non-substituted regions of the xylan backbone, thereby ensuring that most of the formed products have unbranched non-reducing ends, from which (r)BxTW1 (an exo-glycosidase) can initiate its activity. At the same time, the β-xylosidase activity would consume the XOS released by XynM and by this way the product inhibition of the endoxylanases may be prevented (Ball and McCarthy, 1989). In addition, the fact that the final donors in transxylosylation are oligosaccharides, having in many cases polymerization degrees higher than xylobiose, would decrease the number of xylose units lost due to the nature of the double displacement mechanism. Finally, the side α-L-arabinofuranosidase activity displayed by (r)BxTW1 allow expecting good yields for xylans and arabinoxylans exploitation, even in the absence of auxiliary xylanolytic enzymes. Figure 5.1 displays a schematic representation of transxylosylation reactions carried by (r)BxTW1 using xylobiose as donor (Fig. 5.1A) in contrast to the proposed model of enzymatic cascade, which would use xylan and the two xylanolytic enzymes characterized in this work (Fig. 5.1B).

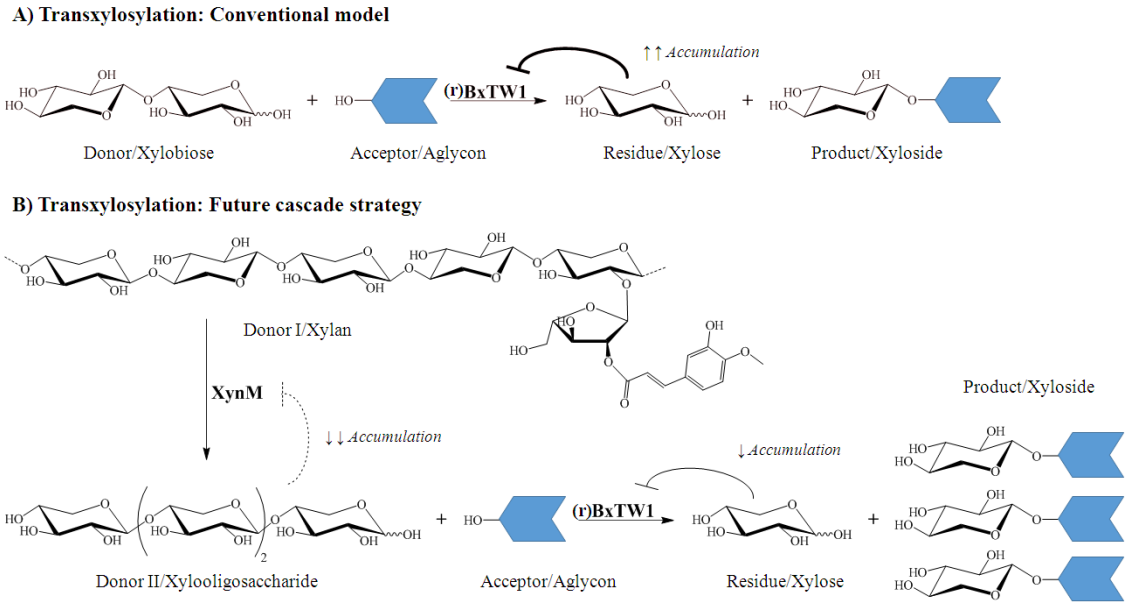


Fig. 5.1. Scheme of a transxylosylation reaction catalyzed by (r)BxTW1 using xylobiose as donor (A) and the proposed model for combining (r)BxTW1 and XynM using xylan as donor (B).

In order to obtain high levels of XynM, allowing the use of this enzyme with a biotechnological purpose, expression studies in *P. pastoris* are being performed. Currently, clones of recombinant yeast displaying activity are already available, which will permit to validate the proposed approach.

6. CONCLUSIONES

CONCLUSIONS

1. El ascomiceto *T. amestolkiae*, un hongo aislado de residuos de cereales, secreta los máximos niveles de actividad xilanolítica en un medio basal con xilano de haya como fuente de carbono. A partir de los sobrenadantes de estos cultivos se aislaron dos nuevas hemicelulasas, una β-xilosidasa perteneciente a la familia GH3 y una endo-β-1,4-xilanasa de la GH11.

2. La β-xilosidasa, BxTW1, se caracteriza por tener una estructura cuaternaria dimérica y ser estable en un amplio rango de pH y en presencia de algunos metales pesados, como el Cu^{2+}. A nivel cinético, muestra la mayor eficacia catalítica descrita hasta la fecha en la hidrólisis de xilooligosacáridos para una β-xilosidasa.

3. La levadura *P. pastoris* resultó ser un buen sistema de expresión para BxTW1. Aunque los cambios en el patrón de glicosilación pudieron afectar a algunas propiedades de esta β-xilosidasa, su producción se incrementó y el proceso de purificación fue más sencillo, observándose una importante mejora en el rendimiento final respecto a la enzima nativa.

4. Tanto la enzima nativa BxTW1 como la forma recombinante, tienen la capacidad de catalizar reacciones de transxilosilación de forma regioselectiva y eficiente. La enzima destaca por tener una amplia versatilidad de aceptores, entre los que se incluyen compuestos aromáticos de estructura compleja.

5. La síntesis del antiproliferativo selectivo 2-(6-hidroxinaftil) β-D-xilopiranósido, es catalizada por rBxTW1 utilizando xilobiosa como donador y 2,6-dihidroxinaftaleno como aceptor. Este proceso, si bien presenta menores rendimientos que la aproximación química convencional, tiene la ventaja de ser respetuoso con el medio ambiente.

6. El 3,4-dihidroxifenil-etil-*O*-β-D-xilopiranósido, representa el primer xilósido descrito del antioxidante hidroxitirosol y fue obtenido mediante transxilosilación catalizada por rBxTW1. El nuevo compuesto presenta propiedades antiinflamatorias similares a las del aglicón, pero mayor poder neuroprotector.

7. La endoxilanasa de *T. amestolkiae*, XynM, posee baja masa molecular y alta selectividad, rasgos típicamente asociados a las glicosidasas de la familia GH11. Su caracterización reveló propiedades similares a las de otras endoxilanasas de los géneros *Penicillium* y *Talaromyces*, aunque destacó su alta tolerancia al Cu^{2+}.

8. XynM es efectiva en la producción de xilooligosacáridos a partir de xilano y los XOS obtenidos de xilano de abedul muestran un efecto prebiótico claro *in vitro*. Los ensayos de esta mezcla en fermentaciones fecales utilizando heces de bebé lactante demostraron actividad bifidogénica e indujeron el crecimiento de *Staphylococcus hominis*, considerado un organismo potencialmente probiótico.

9. Este estudio, junto con los realizados anteriormente sobre el potencial celulolítico de *T. amestolkiae*, ponen de manifiesto que este hongo es un organismo de interés para producir enzimas con gran potencial biotecnológico para la valorización de la biomasa lignocelulósica.

1. The ascomycete *Talaromyces amestolkiae*, a fungus isolated from cereal residues, displays the maximal production of xylanolytic enzymes in a basal medium using beechwood xylan as carbon source. Two novel hemicellulases were isolated from its culture supernatants, a β-xylosidase belonging to the GH3 family and a GH11 endo-β-1,4-xylanase.

2. The β-xylosidase BxTW1 is a dimer, showing high stability in a wide pH range and in the presence of some heavy metals as Cu^{2+}. At the kinetic level, it shows the highest catalytic efficiency described to date in the hydrolysis of xylooligosaccharides for a β-xylosidase.

3. The yeast *Pichia pastoris* was determined to be a suitable system for the heterologous expression of BxTW1. Although changes in the glycosylation pattern seem to affect some β-xylosidase properties, its production is increased and purification becomes simpler, remarkably enhancing the yields of the final process in comparison to the native enzyme.

4. BxTW1, both native and recombinant, catalyzes transxylosylation reactions in a regioselective and efficient way. The acceptor versatility of the enzyme is outstanding and includes complex aromatic compounds.

5. The synthesis of the selective antiproliferative agent 2-(6-hydroxynaphtyl) β-D-xylopyranoside is catalyzed by rBxTW1, using xylobiose as donor and 2,6-dihydroxynaphtalene as acceptor. This process, in spite of showing lower yields than the conventional chemical approach, has the advantage of being environmentally-friendly.

6. 3,4-dihydroxyphenyl-ethyl-*O*-β-D-xylopyranoside is the first reported xyloside obtained from hydroxytyrosol, and it was synthesized by transxylosylation using rBxTW1. The novel compound displays anti-inflammatory properties similar to the aglycon, but a superior neuroprotective effect.

7. The endoxylanase from *T. amestolkiae*, XynM, shows a low molecular mass and high selectivity, properties typically associated to the GH11 family. Its characterization revealed similar properties to those of other xylanases from *Penicillium* and *Talaromyces*, although its high tolerance to Cu^{2+} must be highlighted.

8. XynM efficiently produced xylooligosaccharides from xylan and the obtained birchwood XOS display a clear prebiotic effect *in vitro*. The assays performed on fecal-fermentations using feces from a breast fed

infant demonstrated the bifidogenic activity of the XOS mixture, which also promoted the growth of *Staphylococcus hominis*, considered a potentially prebiotic organism.

9. This study, together with others previously developed on the cellulolytic potential of *T. amestolkiae*, reveals the interest of this fungus as source of enzymes with great potential for valorization of lignocellulosic biomass.

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